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### REMARKS

Applicants thank the Examiner for his review of the instant application. Claims 4-5 have been canceled without prejudice to, or disclaimer of, the subject matter contained therein. Applicants maintain that the cancellation of a claim makes no admission as to its patentability and reserve the right to pursue the subject matter of the cancelled claim in this or any other patent application. Applicants also amend Claim 12 to depend from Claim 6, rather than canceled Claim 4.

For the reasons stated below, the rejections of the presently pending claims are respectfully traversed. Claims 6-17 are presented for examination.

#### Priority

The PTO asserts that because the disclosure of PCT/US00/23328 is not enabling for the instant invention, the filing date of the present application, May 2, 2002, is considered the priority date. For the reasons of record, Applicants maintain that the present application is entitled to at least the priority date of August 24, 2000.

#### Rejection Under 35 U.S.C. §101

The PTO maintains its rejection of pending Claims 6-17 under 35 U.S.C. § 101 as lacking utility for the reasons set forth in the previous Office Actions. The PTO states that the specification discloses that the PRO1277 polynucleotide is more highly expressed in normal esophageal and skin tissue as compared to esophageal and melanoma tumor tissue, respectively, and that Applicants have asserted the use of the molecule for diagnosis. However, the PTO rejects this utility, stating that "[t]here is no further supporting evidence to indicate that the polypeptide encoded by the polynucleotide of the instant invention is also differentially expressed in normal tissue compared to the tumor tissue and as such one of skill in the art would conclude that it is not supported by a substantial asserted utility or a well-established utility." *Final Office Action* at 3-4.

Applicants incorporate by reference their previously submitted arguments, and for the reasons of record assert that the specification contains a disclosure of utility which corresponds in scope to the subject matter sought to be patented and therefore must be taken as sufficient to satisfy the utility requirement of 35 U.S.C. § 101. Applicants also submit that for reasons of

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record, the PTO has not met its burden of providing evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility. However even if the PTO has met its initial burden, Applicants' rebuttal evidence previously submitted and additional evidence submitted herewith is sufficient to prove that it is **more likely than not** that a person of skill in the art would be convinced, **to a reasonable probability**, that the asserted utility is true. As stated previously, Applicants' evidence need not be direct evidence, so long as there is a reasonable correlation between the evidence and the asserted utility. **The standard is not absolute certainty.**

### Substantial Utility

#### Summary of Applicants' Arguments and the PTO's Response

Applicants' asserted utility rests on the following argument:

1. Applicants have provided reliable evidence that mRNA for the PRO1277 polypeptide is expressed at least two-fold higher in normal esophageal and skin tissue as compared to esophageal and melanoma tumor tissue, respectively, and the PTO has accepted that PRO1277 nucleic acids have utility and are enabled as diagnostic tools;
2. Applicants assert that it is well-established in the art that a change in the level of mRNA for a particular protein, *e.g.* an decrease, generally leads to a corresponding change in the level of the encoded protein, *e.g.* an decrease;
3. Given the differential expression of the PRO1277 mRNA in esophageal and melanoma tumors as compared to their normal tissue counterparts, it is more likely than not that the PRO1277 polypeptide is also differentially expressed in esophageal and melanoma tumors as compared to their normal tissue counterparts, making the claimed polypeptides useful as diagnostic tools, alone or in combination with other diagnostic tools.

Applicants understand the PTO to be making two arguments in response to Applicants' asserted utility:

1. The PTO challenges the reliability of the evidence reported in Example 18, stating for example that there is no guidance in the specification as to how high the expression levels are, and that the literature cautions against drawing conclusions based on small changes in transcript expression levels between normal and cancerous tissue, citing Hu *et al.* for support;

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2. The PTO cites Haynes *et al.* (Electrophoresis, 1998; 19(11):1862-71), Chen *et al.* (Mol. and Cell. Proteomics, 2002; 1:304-313) and Gygi *et al.* (Mol. and Cell. Bio. 1999; 19(3):1720-30) to support its assertion that polypeptide levels cannot be accurately predicted from mRNA levels. Therefore, further research needs to be done to determine if the increase or decrease in PRO1277 cDNA expression supports a role for the peptide in cancerous tissue.

Applicants respectfully submit that in light of all of the evidence, the PTO's arguments are not adequate to support the utility rejection of the claimed invention under 35 U.S.C. § 101.

*The PTO has Acknowledged that the Data Reporting Differential Expression of PRO1277 mRNA is Sufficient to Provide Utility for the mRNA as a Diagnostic Tool*

Applicants first address the PTO's argument that the evidence of differential expression of the gene encoding the PRO1277 polypeptide in esophageal and melanoma tumors is insufficient, and that the literature cautions against drawing conclusions based on small changes in transcript expression levels between normal and cancerous tissue.

Applicants note that in the closely related application Serial No. 10/063,688, directed to nucleic acids related to SEQ ID NO:33 which encodes the PRO1277 polypeptide, the PTO has acknowledged that the nucleic acids have utility. *See Office Action for Application 10/063,688 dated 9/15/2005 at 2.* In that case, the exact same data from Example 18 was relied on for utility of the claimed nucleic acids as diagnostic tools for esophageal and melanoma tumors, and the PTO made the same arguments regarding the insufficiency of the data in Example 18 and the cautionary teachings of Hu *et al.* In response to Applicants' arguments to the contrary, the PTO stated "Applicants assertion that the differentially expressed message can be used as a diagnostic tool for stomach and lung [*sic*] tumors is found to be persuasive." *Id.* at 2 (emphasis added). Therefore, Applicants submit that the PTO's rejection of the exact same data in the instant case based on the same arguments of alleged insufficient details or the teaching Hu *et al.* is moot in light of this statement. As such, the data in Example 18 are sufficient to establish utility for the PRO1277 nucleic acids as a diagnostic tool.

In addition to the persuasive reasons articulated in Applicants' arguments of record, the PTO's reliance on Hu is also misplaced because Applicants are not relying on microarray data as discussed in Hu:

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In any microarray experiment, thousands of genes may demonstrate statistically significant expression changes, but only a fraction of these may be relevant to the study. *Hu* at 405, left column, first paragraph (emphasis added).

Instead, Applicants are relying on a more accurate and reliable method of assessing changes in mRNA level, namely quantitative PCR analysis. In a recent study by Kuo *et al.*, (Proteomics 5(4):894-906 (2005)), the authors used microarray analysis combined with proteomic analysis using two-dimensional gel electrophoresis to examine changes in gene expression in leukemia cell lines. The authors report that “[c]omparison of microarray and proteomic expression profiles showed poor correlation. Use of more reliable and sensitive analyses, such as reverse transcriptase polymerase chain reaction [RT-PCR], Western blotting and functional assays, on several genes and proteins, nonetheless, confirmed that there is indeed good correlation between mRNA and protein expression.” Kuo *et al.* at Abstract (emphasis added) (attached as Exhibit 1). Thus, even if accurate, Hu’s statements regarding microarray studies are not relevant to the instant application which does not rely on microarray data.

In conclusion, Applicants submit that the evidence reported in Example 18, supported by the first Grimaldi Declaration, establish that there is at least a two-fold difference in PRO1277 mRNA between esophageal and melanoma tumors as compared to their normal tissue counterparts. The PTO has accepted that the data in Example 18 are sufficient to establish utility for the nucleic acids encoding the PRO1277 polypeptide as diagnostic tools, and therefore any challenge to the sufficiency of the data with respect to the utility of the nucleic acid is inappropriate. Therefore, the only issue which remains is whether the data in Example 18 regarding differential expression of the PRO1277 mRNA are reasonably correlated with differential expression of the PRO1277 polypeptide such that the claimed polypeptides have utility as diagnostic tools as well. As discussed below, even if the PTO has established a reasonable doubt regarding Applicants’ assertion that they are reasonably correlated, Applicants’ overwhelming rebuttal evidence is more than sufficient to establish that changes in mRNA level lead to corresponding changes in protein level.



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*The PTO's Evidence is Not Relevant to Determining Whether a Change in mRNA Level for a Particular Gene leads to a Corresponding Change in the Level of the Encoded Protein*

Applicants turn next to the second portion of their argument in support of their asserted utility – that it is well-established in the art that a change in the level of mRNA encoding a particular protein generally leads to a corresponding change in the level of the encoded protein; given Applicants' evidence of differential expression of the mRNA for the PRO1277 polypeptide in esophageal and melanoma tumors, it is likely that the PRO1277 polypeptide is also differentially expressed; and proteins differentially expressed in certain tumors have utility as diagnostic tools.

In response to Applicants' assertion, the PTO cites Haynes *et al.* (Electrophoresis, 1998; 19(11):1862-71), Chen *et al.* (Mol. and Cell. Proteomics, 2002; 1:304-313) and Gygi *et al.* (Mol. and Cell. Bio. 1999; 19(3):1720-30) as support for its argument that "polypeptide levels cannot be accurately predicted from mRNA levels." The PTO also makes the argument that "it is noted that the features upon which applicant relies (i.e., changes in message levels are correlated to protein levels) are not recited in the rejected claim(s). Although claims are interpreted in light of the specification, limitations from the specification are not read into the claims." *Final Office Action* at 6.

As to the PTO's argument that the claims do not recite the feature being relied on, this argument fails because it misstates the law. Applicants do not need to recite any additional limitations to make arguments about what one of skill in the art would have believed regarding the utility of the claimed polypeptides. As explained above, Applicants are arguing that given the differential expression of the PRO1277 mRNA, one of skill in the art would believe that the PRO1277 polypeptide is also differentially expressed, and therefore one of skill in the art would believe that the asserted utility is more likely than not true. Applicants rely on the assertion that it is well established in the art that changes in mRNA level lead to changes in protein level to support an asserted utility for the claimed polypeptides. This assertion does not need to be recited in the claims to be relied on to support Applicants' utility. The PTO cannot cite any statute, case law or M.P.E.P. section to support its argument to the contrary because no such requirement exists.

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As to the PTO's cited references, Applicants have previously discussed at length why the Haynes, Chen and Gygi references are not relevant to the issue of whether changes in mRNA level for a particular gene lead to changes in protein level. Applicants incorporate by reference the previous arguments, and will not repeat them here.

However, in an attempt to illustrate why references which relate to static global levels of mRNA and protein across different genes are not relevant to this issue, Applicants offer the following illustration and analogy with the understanding that like all illustrations and analogies, they are not perfect and therefore do not represent any admissions or binding statements regarding Applicants' disclosure or invention.

Haynes, Gygi, and portions of Chen all discuss whether there is a correlation between the static level of mRNAs and proteins globally, *i.e.* across different genes. This is equivalent to conducting a hypothetical Experiment 1, where a particular cell type has 100 copies of mRNA for gene X, 200 copies of mRNA for gene Y, and 400 copies of mRNA for gene Z. If there is a global correlation between static mRNA levels and protein levels across genes, the ratio of the amount of proteins X:Y:Z would be approximately 1:2:4. This is essentially what the cited references examined.

In contrast, Applicants are relying on a correlation between changes in mRNA level for a particular gene leading to a corresponding change in the level of the encoded protein. For example, in hypothetical Experiment 2, if gene X has 200 copies of mRNA per cell in condition A (*e.g.* normal), and 100 copies of mRNA for gene X in condition B (*e.g.* tumor), the ratio of the amount of protein X in condition A:B would be approximately 2:1, such that there is a correlation between the change in the level of mRNA and protein for a particular gene.

The PTO argues that because there is no correlation between static levels of mRNA and protein across genes, as illustrated by Experiment 1, one of skill in the art would not expect an increase or decrease in the amount of mRNA for a particular gene to result in a corresponding change in the amount of the encoded protein, as illustrated in Experiment 2. This is simply wrong.

For example, Haynes reports that the amount of protein produced by similar levels of mRNA varied by as much as fifty-fold, and that similar amounts of protein were sustained by amounts of mRNA that varied by as much as forty-fold. *Haynes* at 1863, first full paragraph.

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Based on these results, Haynes concludes that “protein levels cannot be accurately predicted from the level of the corresponding mRNA transcript.” *Id.*

This is analogous to a finding that on one gallon of gas, a hybrid car can travel 70 miles but a large truck can only travel 5 miles, or that to travel 70 miles, a hybrid car requires 1 gallon of gas, but a large truck requires 14 gallons. That is to say, there are many things which affect the fuel efficiency of an automobile. Based on these observations, one could conclude that given the lack of correlation between the amount of gas in an automobile and the distance it travels, one cannot predict how far an automobile will travel based on the amount of gas in the tank.

Even if true, Haynes’ data and conclusions are irrelevant to Applicants’ assertion, which is that increasing or decreasing the amount of mRNA for a particular gene will result in a corresponding increase or decrease in the amount of the encoded protein. This is analogous to increasing or decreasing the amount of gas in an automobile – it will travel farther if you add more gas, and not as far with less. The fact that there are many things which affect fuel efficiency and therefore you cannot predict how far an automobile will travel without knowing if it is a hybrid or a large truck is irrelevant – both a hybrid and a truck travel farther on more gas, and not as far on less.

Similarly, Chen *et al.* report that plotting the level of mRNA for a particular gene against the level of the corresponding protein as measured across numerous samples, they found a lack of correlation for most genes studied. *Chen* at Abstract. However, with the exception of three genes reported in Figures 2A-2C, Chen does not indicate whether the level of mRNA varied significantly across samples, and Chen did not select samples or genes which were expected to vary across samples (*e.g.* normal versus tumor). Therefore, it is not known if Chen examined changes in mRNA level, or if the level of mRNA was unchanged. Therefore, the relevance of Chen’s finding to Applicants’ asserted correlation between changes in mRNA and protein is not known.

By analogy, if a person drives a particular car as far as possible on 5 gallons of gas 20 different times, and then plots the amount of gas against the distance driven, a lack of correlation between the amount of gas and distance is meaningless, and merely reflects systematic error in measuring the amount of gas and distance driven. Only if substantially different amounts of gas

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were plotted against their respective distances can you answer the question of whether increasing or decreasing the amount of gas results in increasing or decreasing the distance driven.

Applicants emphasize, and the PTO will recognize, that these are simplified illustrations to demonstrate the difference between the two issues being examined. However, these illustrations make clear that even if there is no correlation in the first experiment looking at static levels of mRNA and protein across genes, there can still be a correlation between changes in mRNA and protein for a particular gene as examined in the second experiment. As these illustrations make clear, the PTO's evidence simply is not relevant to answering the question of whether it is **more likely than not** that a person of skill in the art would be convinced, to a **reasonable probability**, that the asserted utility is true.

*Applicants' Evidence Establishes that a Change in mRNA Level for a Particular Gene lead to Corresponding Change in the Level of the Encoded Protein*

In support of the assertion that changes in mRNA are positively correlated to changes in protein levels, Applicants previously submitted a copy of a second Declaration by J. Christopher Grimaldi, a copy of the declaration of Paul Polakis, Ph.D., excerpts from the Molecular Biology of the Cell, a leading textbook in the field (Bruce Alberts, *et al.*, Molecular Biology of the Cell (3<sup>rd</sup> ed. 1994) and (4<sup>th</sup> ed. 2002), excerpts from the textbook, Genes VI, (Benjamin Lewin, Genes VI (1997)), a reference by Zhigang *et al.* (World Journal of Surgical Oncology, 2004; 2:13), and a reference by Meric *et al.*, Molecular Cancer Therapeutics, 2002; 1:971-979). The details of the teachings of these declarations and references, and how they support Applicants' asserted utility, are of record and will not be repeated here.

Applicants submit herewith a copy of a second Declaration by Dr. Polakis (attached as Exhibit 2) that presents evidentiary data in Exhibit B. Exhibit B of the Declaration identifies 28 gene transcripts out of 31 gene transcripts (i.e., greater than 90%) that showed good correlation between tumor mRNA and tumor protein levels. As Dr. Polakis' second Declaration says "[a]s such, in the cases where we have been able to quantitatively measure both (i) mRNA and (ii) protein levels in both (i) tumor tissue and (ii) normal tissue, we have observed that in the vast majority of cases, there is a very strong correlation between increases in mRNA expression and

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increases in the level of protein encoded by that mRNA.” Accordingly, Dr. Polakis has provided the facts to enable the PTO to draw independent conclusions.

In addition to the supporting references previously submitted by Applicants, Applicants submit the following references to further support the assertion that changes in mRNA levels generally lead to corresponding changes in the level of the encoded polypeptide.

In a comprehensive study by Orntoft *et al.* (Mol. Cell. Proteomics. 2002; 1(1):37-45) (previously submitted with IDS, attached hereto as Exhibit 3), the authors examined gene amplification, mRNA expression level, and protein expression in pairs of non-invasive and invasive human bladder tumors. *Id.* at Abstract. The authors examined 40 well resolved abundant known proteins, and found that “[i]n general there was a highly significant correlation ( $p < 0.005$ ) between mRNA and protein alterations. Only one gene showed disagreement between transcript alteration and protein alteration.” *Id.* at 42, col. 2. The alternations in mRNA and protein included both increases and decreases. *Id.* at 43, Table II. Clearly, a correlation in 39 of 40 genes examined supports Applicants’ assertion that changes in mRNA level generally lead to corresponding changes in protein level.

In a study by Wang *et al.* (Urol. Res. 2000; 28(5):308-15) (abstract attached as Exhibit 4) the authors report that down-regulation of E-cadherin protein has been shown in various human tumors. *Id.* at Abstract. In the reported study, the authors examined the expression of cadherins and associated catenins at the mRNA level in paired tumor and nonneoplastic primary prostate cultures. They report that “[s]ix of seven cases of neoplastic cultures showed moderately-to-markedly decreased levels of E-cadherin and P-cadherin mRNA. Similar losses of alpha-catenin and beta-catenin mRNA were also observed.” *Id.* As Applicants’ assertion would predict, the authors state that the mRNA measures showed “good correlation” with the results from protein measures. The authors conclude by stating that “this paper presents a coordinated down-regulation in the expression of E-cadherin and associated catenins at the mRNA and protein level in most of the cases studied.” *Id.*

In a more recent study by Munaut *et al.* (Int. J. Cancer. 2003; 106(6):848-55) (abstract attached as Exhibit 5) the authors report that vascular endothelial growth factor (VEGF) is expressed in 64-95% of glioblastomas (GBMs), and that VEGF receptors (VEGFR-1, its soluble form sVEGFR-1, VEGFR-2 and neuropilin-1) are expressed predominantly by endothelial cells.

*Id.* at Abstract. The authors explain that infiltrating tumor cells and newly-formed capillaries progress through the extracellular matrix by local proteolysis involving matrix metalloproteinases (MMPs). In the present study, the authors “used quantitative RT-PCR, Western blot, gelatin zymography and immunohistochemistry to study the expression of VEGF, VEGFR-1, VEGFR-2, sVEGFR-1, neuropilin-1, MT1-MMP, MMP-2, MMP-9 and TIMP-2 in 20 human GBMs and 5 normal brains. The expression of these MMPs was markedly increased in most GBMs with excellent correlation between mRNA and protein levels.” *Id.* Thus, the results support Applicants’ assertion that changes in mRNA level lead to corresponding changes in protein level.

In another recent study, Hui *et al.* (Leuk. Lymphoma. 2003; 44(8):1385-94 (abstract attached as Exhibit 6) used real-time quantitative PCR and immunohistochemistry to evaluate cyclin D1 mRNA and protein expression levels in mantle cell lymphoma (MCL). *Id.* at Abstract. The authors report that seven of nine cases of possible MCL showed overexpression of cyclin D1 mRNA, while two cases showed no cyclin D1 mRNA increase. *Id.* Similarly, “[s]ix of the seven cyclin D1 mRNA overexpressing cases showed increased cyclin D1 protein on tissue array immunohistochemistry; one was technically suboptimal.” *Id.* The authors conclude that the study “demonstrates good correlation and comparability between measure of cyclin D1 mRNA ... and cyclin D1 protein.” *Id.* Thus, this reference supports Applicants’ assertion.

In a recent study by Khal *et al.* (Int. J. Biochem. Cell Biol. 2005; 37(10):2196-206) (abstract attached as Exhibit 7) the authors report that atrophy of skeletal muscle is common in patients with cancer and results in increased morbidity and mortality. *Id.* at Abstract. To further understand the underlying mechanism, the authors studied the expression of the ubiquitin-proteasome pathway in cancer patient muscle using a competitive RT-PCR to measure expression of mRNA for proteasome subunits C2 and C5, while protein expression was determined by western blotting. “Overall, both C2 and C5 gene expression was increased by about three-fold in skeletal muscle of cachectic cancer patients (average weight loss 14.5+/-2.5%), compared with that in patients without weight loss, with or without cancer. ... There was a good correlation between expression of proteasome 20Salpha subunits, detected by western blotting, and C2 and C5 mRNA, showing that increased gene expression resulted in increased

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protein synthesis.” These findings support Applicants’ assertion that changes in mRNA level lead to changes in protein level.

Maruyama *et al.* (Am. J. Patho. 1999; 155(3):815-22) (abstract attached as Exhibit 8) investigated the expression of three Id proteins (Id-1, Id-2 and Id-3) in normal pancreas, in pancreatic cancer and in chronic pancreatitis (CP). The authors report that pancreatic cancer cell lines frequently coexpressed all three Ids, “exhibiting good correlation between Id mRNA and protein levels.” *Id.* at Abstract. In addition, the authors teach that all three Id mRNA levels were expressed at high levels in pancreatic cancer samples compared to normal or CP samples. At the protein level, Id-1 and Id-2 staining was faint in normal tissue, while Id-3 ranged from weak to strong. In contrast, in the cancer tissues “many of the cancer cells exhibited abundant Id-1, Id-2, and Id-3 immunoreactivity,” and Id-1 and Id-2 protein was increased significantly in the cancer cells by comparison to the respective controls, mirroring the overexpression at the mRNA level. Thus, the authors report that in both cell lines and tissue samples, increased mRNA levels leads to an increase in protein overexpression, supporting Applicants’ assertion.

Support for Applicants’ assertion is also found in an article by Caberlotto *et al.* (Neurosci. Lett. 1999; 256(3):191-4) (abstract attached as Exhibit 9). In a previous study, the authors investigated alterations of neuropeptide Y (NPY) mRNA expression in the Flinders Sensitive Line rats (FSL), an animal model of depression. *Id.* at Abstract. The authors reported that in the current study, that NPY-like immunoreactivity (NPY-LI) was decreased in the hippocampal CA region, and increased in the arcuate nucleus, and that fluoxetine treatment elevated NPY-LI in the arcuate and anterior cingulate cortex. The authors state that “[t]he results demonstrate a good correlation between NPY peptide and mRNA expression.” Thus, increases and decreases in mRNA levels were reflected in corresponding changes in protein level.

Mizrachi and Shemesh (Biol. Reprod. 1999; 61(3):776-84) (abstract attached as Exhibit 10) investigated their hypothesis that FSH regulates the bovine cervical prostaglandin E(2) (PGE(2)) synthesis that is known to be associated with cervical relaxation and opening at the time of estrus. *Id.* at Abstract. Cervical tissue from pre-estrous/estrous, luteal, and postovulatory cows were examined for the presence of bovine (b) FSH receptor (R) and its corresponding mRNA. The authors report that bFSHR mRNA in the cervix was maximal during pre-estrus/estrus, and that the level of FSHR protein was significantly higher in

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pre-estrous/estrous cervix than in other cervical tissues. *Id.* The authors state that “[t]here was a good correlation between the 75-kDa protein expression and its corresponding transcript of 2.55 kb throughout the estrous cycle as described by Northern blot analysis as well as RT-PCR.” *Id.* Thus, changes in the level of mRNA for bFSHR led to corresponding changes in FSHR protein levels, a result which supports Applicants’ assertion.

In a study by Stein *et al.* (J. Urol. 2000; 164(3 Pt 2):1026-30) (abstract attached as Exhibit 11), the authors studied the role of the regulation of calcium ion homeostasis in smooth muscle contractility. *Id.* at Abstract. The authors investigated the correlation between sarcoplasmic endoplasmic reticulum, calcium, magnesium, adenosine triphosphatase (SERCA) protein and gene expression, and the contractile properties in the same bladder. Partial bladder outlet obstructions were created in adult New Zealand white rabbits, which were divided into control, sham operated and obstructed groups. Stein *et al.* report that “[t]he relative intensities of signals for the Western [protein] and Northern [mRNA] blots demonstrated a strong correlation between protein and gene expression. ... The loss of SERCA protein expression is mediated by down-regulation in gene expression in the same bladder.” *Id.* This report supports Applicants’ assertion that changes in mRNA level, e.g. a decrease, lead to a corresponding change in the level of the encoded protein, e.g. a decrease.

In an article by Gou and Xie (Zhonghua Jie He He Hu Xi Za Zhi. 2002; 25(6):337-40) (abstract attached as Exhibit 12) the authors investigated the expression of macrophage migration inhibitory factor (MIF) in human acute respiratory distress syndrome(ARDS) by examining the expression of MIF mRNA and protein in lung tissue in ARDS and normal persons. *Id.* at Abstract. The authors report “undetectable or weak MIF mRNA and protein expression in normal lungs. In contrast, there was marked upregulation of MIF mRNA and protein expression in the ARDS lungs.” *Id.* This is consistent with Applicants’ assertion that a change in mRNA for a particular gene, e.g. an increase, generally leads to a corresponding change in the level of protein expression, e.g. an increase.

These studies are representative of numerous published studies which support Applicants’ assertion that changes in mRNA level generally lead to corresponding changes in the level of the expressed protein. Applicants submit herewith an additional 70 references (abstracts attached as Exhibit 13) which support Applicants’ assertion.



In addition to these supporting references, Applicants also submit herewith additional references which offer indirect support of Applicants' asserted utility. As discussed in detail above, Applicants have challenged the relevance of references such as Haynes *et al.*, Gygi *et al.*, and Chen *et al.* which do not attempt to examine the correlation between a change in mRNA level and a change in the level of the corresponding protein level. Because the PTO continues to rely on these references, Applicants are submitting references which report results that are contrary to the PTO's cited references and offer indirect support for Applicants' asserted utility.

For example, in an article by Futcher *et al.* (Mol. Cell Biol. 1999; 19(11):7357-68) (abstract attached as Exhibit 14) the authors conducted a study of mRNA and protein expression in yeast which was nearly identical to the one conducted by Gygi *et al.* and reported in Haynes *et al.* Contrary to the results of the earlier study by Gygi, Futcher *et al.* report "a good correlation between protein abundance, mRNA abundance, and codon bias." *Id.* at Abstract.

In a study which is more closely related to Applicants' asserted utility, Godbout *et al.* (J. Biol. Chem. 1998; 273(33):21161-8) (abstract attached as Exhibit 15) studied the DEAD box gene, DDX1, in retinoblastoma and neuroblastoma tumor cell lines. The authors report that "there is a good correlation with DDX1 gene copy number, DDX1 transcript levels, and DDX1 protein levels in all cell lines studied." *Id.* Thus, in these cancer cell lines, DDX1 mRNA and protein levels are correlated.

Similarly, in an article by Papotti *et al.* (Virchows Arch. 2002; 440(5):461-75) (abstract attached as Exhibit 16) the authors examined the expression of three somatostatin receptors (SSTR) at the mRNA and protein level in forty-six tumors. *Id.* at Abstract. The authors report a "good correlation between RT-PCR [mRNA level] and IHC [protein level] data on SSTR types 2, 3, and 5." *Id.*

Van der Wilt *et al.* (Eur. J. Cancer. 2003; 39(5):691-7) (abstract attached as Exhibit 17) studied deoxycytidine kinase (dCK) in seven cell lines, sixteen acute myeloid leukemia samples, ten human liver samples, and eleven human liver metastases of colorectal cancer origin. *Id.* at Abstract. The authors report that "enzyme activity and protein expression levels of dCK in cell lines were closely related to the mRNA expression levels" and that there was a "good correlation between the different dCK measurements in malignant cells and tumors." *Id.*

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Grenback *et al.* (Regul. Pept. 2004; 117(2):127-39) (abstract attached as Exhibit 18) studied the level of galanin in human pituitary adenomas using a specific radioimmunoassay. *Id.* at Abstract. The authors report that “[i]n the tumors analyzed with in situ hybridization there was a good correlation between galanin peptide levels and galanin mRNA expression.” *Id.*

Similarly, Shen *et al.* (Blood. 2004; 104(9):2936-9) (abstract attached as Exhibit 19) examined the level of B-cell lymphoma 2 (BCL2) protein expression in germinal center (GC) B-cells and diffuse large B-cell lymphoma (DLBCL). *Id.* at Abstract. The authors report that “GC cells had low expression commensurate with the low protein expression level” and that in DLBCL the level of BCL2 mRNA and protein expression showed “in general, a good correlation.” *Id.*

Likewise, in an article by Fu *et al.* (Blood 2005; 106(13):4315-21) (abstract attached as Exhibit 20) the authors report that six mantle cell lymphomas studied “expressed either cyclin D2 (2 cases) or cyclin D3 (4 cases).” *Id.* at Abstract. “There was a good correlation between cyclin D protein expression and the corresponding mRNA expression levels by gene expression analysis.” *Id.*

These examples are only a few of the many references Applicants could cite in rebuttal to the PTO’s arguments. Applicants submit herewith 26 additional references (abstracts attached as Exhibit 21) which also support Applicants’ assertion in that the references report a correlation between the level of mRNA and corresponding protein, contrary to the assertion of the PTO that mRNA and protein levels are not correlated.

In summary, Applicants submit herewith a total of 113 references in addition to the declarations and references already of record which support Applicants’ asserted utility, either directly or indirectly. These references support the assertion that in general, a change in mRNA expression level for a particular gene leads to a corresponding change in the level of expression of the encoded protein. As Applicants have previously acknowledged, the correlation between changes in mRNA level and protein level is not exact, and there are exceptions (*see, e.g.*, abstracts attached as Exhibit 22). However, Applicants remind the PTO that the asserted utility does not have to be established to a statistical certainty, or beyond a reasonable doubt. *See M.P.E.P.* at § 2107.02, part VII (2004). Therefore, the fact that there are exceptions to the correlation between changes in mRNA and changes in protein does not provide a proper basis for

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rejecting Applicants' asserted utility. Applicants submit that considering the evidence as a whole, with the overwhelming majority of the evidence supporting Applicants' asserted utility, a person of skill in the art would conclude that Applicants' asserted utility is "more likely than not true." *Id.*

In conclusion, Applicants submit that they have offered sufficient evidence to establish that it is more likely than not that one of skill in the art would believe that because the PRO1277 mRNA is differentially expressed in esophageal and melanoma tumors as compared to their normal tissue counterparts, the PRO1277 polypeptide will likewise be differentially expressed in esophageal and melanoma tumors. This differential expression of the PRO1277 polypeptide makes the claimed polypeptides useful as diagnostic tools for cancer, particularly esophageal and melanoma cancer.

### **Specific Utility**

#### **The Asserted Substantial Utilities are Specific to the Claimed Polypeptides**

Applicants next address the PTO's assertion that the asserted utilities are not specific to the claimed polypeptides related to PRO1277. Applicants respectfully disagree.

Specific utility is defined as utility which is "specific to the subject matter claimed," in contrast to "a general utility that would be applicable to the broad class of the invention." M.P.E.P. § 2107.01 I. Applicants submit that the evidence of differential expression of the PRO1277 gene and polypeptide in certain types of tumor cells, along with the declarations and references discussed above, provide a specific utility for the claimed polypeptides.

As discussed above, there are significant data which show that the gene for the PRO1277 polypeptide is differentially expressed by at least two-fold in esophageal and melanoma tumors as compared to their normal tissue counterparts. These data are strong evidence that the PRO1277 gene and polypeptide are associated with esophageal and melanoma tumors. Thus, contrary to the assertions of the PTO, Applicants submit that they have provided evidence associating the PRO1277 gene and polypeptide with a specific disease. The asserted utility for the claimed polypeptides as diagnostic tools for cancer, particularly esophageal and melanoma tumors, is a specific utility – it is not a general utility that would apply to the broad class of polypeptides.

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### Utility – Conclusion

Applicants remind the PTO that the evidence supporting utility does not need to be direct evidence, nor does it need to provide an exact correlation between the submitted evidence and the asserted utility. Instead, evidence which is “reasonably” correlated with the asserted utility is sufficient. *See Fujikawa v. Wattanasin*, 93 F.3d 1559, 1565, 39 U.S.P.Q. 2d 1895 (Fed. Cir. 1996) (“a ‘rigorous correlation’ need not be shown in order to establish practical utility; ‘reasonable correlation’ suffices”); *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 U.S.P.Q. 739 (Fed. Cir. 1985) (same); *Nelson v. Bowler*, 626 F.2d 853, 857, 206 U.S.P.Q. 881 (C.C.P.A. 1980) (same). In addition, utility need only be shown to be “more likely than not true,” not to a statistical certainty. *M.P.E.P.* at § 2107.02, part VII (2004). Considering the evidence as a whole in light of the relevant standards for establishing utility, Applicants have established at least one specific, substantial, and credible utility. In view of the above, Applicants respectfully request that the PTO reconsider and withdraw the utility rejection under 35 U.S.C. §101.

### **Rejections under 35 U.S.C. § 112, first paragraph – Enablement**

The PTO also maintains its rejection of pending Claims 6-17 under 35 U.S.C. § 112, first paragraph. Specifically, the PTO asserts that because the claimed invention is not supported by either a specific or substantial asserted utility or a well established utility, one skilled in the art would not know how to use the claimed invention. *Final Office Action* at 3. In addition, the PTO makes the conclusory statement that “[e]ven if the specification taught how to use the PRO1277 polypeptide (SEQ ID NO:34), enablement would not be commensurate in scope with Claims 4-5, and 12-17, which encompass % variants of SEQ ID NO:34 (claims 4-5, for example), and various fragments of SEQ ID NO: 28 (claims 4-5, 14 and 15 for example).” *Final Office Action* at 15 (emphasis added).

Applicants note that none of the pending claims recite SEQ ID NO:28, and therefore Applicants assume that the underlined portion of the above quote from the pending Office Action is merely a typographical mistake.

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*The PTO has Failed to Establish a Reasonable Basis to Question the Enablement of the Pending Claims*

The PTO has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *See M.P.E.P.* § 2164.04. It is incumbent for the PTO “to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement.” *Id.* (quoting *In re Marzocchi*, 439 F.2d 220, 224, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971)). This can be done “by making specific findings of fact, supported by the evidence, and then drawing conclusions based on these findings of fact.” *Id.*

As an initial matter, Applicants submit that in the discussion of the 35 U.S.C. § 101 rejection above, Applicants have established a substantial, specific, and credible utility for the claimed polypeptides. To the extent that the enablement rejection is based on a lack of utility, Applicants respectfully request that the PTO reconsider and withdraw the enablement rejection under 35 U.S.C. §112.

Turning to the PTO’s other arguments, in the most recent final Office Action, the PTO makes the conclusory statement that enablement is not commensurate in scope with claims “because there is no structural or functional information provided in the specification.” *Final Office Action* at 15. In addition, the PTO summarily states that:

In addition, the lack of direction/guidance presented in the specification regarding which variants of polypeptides of SEQ ID NO:34 would retain the desired activity, the complex nature of the invention, the state of the prior art establishing that biological activity cannot be predicted based on structural similarity, the absence of working examples directed to variants and the breath of claims, undue experimentation would be required of the skilled artisan to make and/or use the claimed invention in its full scope. *Id.* at 15.

The PTO discusses the amendment to Claims 4-5, but offers no discussion of the structure-function limitation “wherein said isolated polypeptide or a fragment thereof can be used to generate an antibody which can be used to specifically detect the polypeptide of SEQ ID NO: 28 in rectal or kidney tissue samples” found in Claims 14-17. *See Office Action* at 15-16.

The PTO’s statements fail to establish a reasonable basis to question the enablement provided for the claimed invention. *See M.P.E.P.* § 2164.04. It is incumbent for the PTO “to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to

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back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement.” *Id.* (quoting *In re Marzocchi*, 439 F.2d 220, 224, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971). This can be done “by making specific findings of fact, supported by the evidence, and then drawing conclusions based on these findings of fact.” *Id.* The PTO has failed to make any specific findings of fact, or back up its assertions with any acceptable evidence or reasoning.

As an initial matter, Applicants note that pending Claims 6 and 12-13 do not recite percent amino acid sequence identity as a limitation. These claims are directed to peptides of the disclosed sequence, and fusion proteins thereof which would be optimal, for example, in making antibodies. Therefore, any arguments based on a failure to enable variants are not applicable to pending Claims 12-13.

As to the remaining claims, the specification teaches in detail how to make the claimed polypeptides, including variants thereof, and antibodies which specifically bind PRO1277. Likewise, the specification provides sufficient guidance as to how to use the claimed polypeptides. Thus, contrary to the PTO’s statement, there is significant guidance how to make and use the claimed polypeptides. In addition, as the disclosure and references cited in the specification make clear, the production of polypeptides, polypeptide variants, and specific antibodies is a predictable and well established aspect of the biological sciences. *See, e.g., In re Wands*, 858 F.2d 731, 8 U.S.P.Q. 2d 1400 (Fed. Cir. 1988) (reversing the Board’s decision of non-enablement and holding that as of 1980, undue experimentation was not required to make high-affinity monoclonal antibodies to a target peptide); *Sutcliffe et al.*, *Science* (1983) 219:660-666 at 661-662 (teaching that “by following simple rules, one can in general select peptides that will elicit antibodies reactive with intact proteins”) (attached as Exhibit 23).

It is important to note that the PTO has not addressed the structure-function limitation found in Claims 14-17 in any of the Office Actions. The PTO has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention. *See M.P.E.P.* § 2164.04. It is incumbent for the PTO “to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement.” *Id.* (quoting *In re Marzocchi*, 439 F.2d 220, 224, 169 U.S.P.Q. 367, 370 (C.C.P.A. 1971). Clearly, in not

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addressing Claims 14-17, the PTO has failed to meet this burden and the presumption of enablement remains undisputed.

In conclusion, the PTO's rejection based on lack of utility has been addressed above, and the PTO has otherwise failed to meet its burden to establish a reasonable basis to question the enablement provided for the claimed invention – unsupported conclusory statements are simply not sufficient. Given the skill in the art and the disclosure of how to make and use the claimed polypeptides, Applicants request that the PTO reconsider and withdraw its rejection under 35 U.S.C. § 112, first paragraph.

**Rejection under 35 U.S.C. §112, first paragraph – Written Description**

The PTO maintains the rejection of pending Claims 12-17 under 35 U.S.C. § 112, first paragraph, as failing to satisfy the written description requirement for the reasons set forth in the previous Office Actions. Briefly, the PTO asserts the “Applicants have not described or shown possession of all polypeptides that have 95-99% homology to SEQ ID NO:34 or fragments recited and still retain the function of SEQ ID NO: 34.” *Final Office Action* at 17 (emphasis added).

**The PTO has Failed to Meet Its Initial Burden of Rebutting the Presumption that the Pending Claims are Adequately Described**

To overcome the presumption that the claimed subject matter is adequately described, the PTO must present “evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims. *Wertheim*, 541 F.2d at 263, 191 U.S.P.Q. at 97.” *M.P.E.P.* § 2163.04. During the course of prosecution, the PTO has made essentially two arguments in an attempt to rebut this presumption.

First, the PTO has asserted that that the claims “are drawn to polynucleotides [*sic*] having at least 80%, 85%, 95% or 99% sequence identity with a particular disclosed sequence. The claims do not require that the claimed polypeptide possess any particular biological activity, nor any particular conserved structure, or other disclosed distinguishing feature.” *First Office Action* at 10. Second, the PTO has asserted that “even a very skilled artisan could not envision the detailed chemical structure of all or a significant number of encompassed PRO1277

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polypeptides, and therefore, would not know how to make and use them.” *Final Office Action* at 18. The PTO also discusses the particular amendment to Claims 4 and 5, but these arguments are moot in light of Applicants canceling Claims 4-5 and amending Claim 12 to depend from Claim 6. *Final Office Action* at 17-18.

The PTO has failed to meet its initial burden of rebutting the presumption that the written description is adequate because nowhere in any Office Action does the PTO specifically address Claims 14-17, or explain how its arguments apply to pending Claims 6 and 12-13 which are not directed to variant polypeptides. Instead, the PTO’s arguments address only Claims 4-5.

*The Pending Claims are Adequately Described*

As mentioned above, pending Claims 6 and 12-13 do not recite percent sequence identity, and therefore are adequately described by the disclosure of Figure 34, SEQ ID NO:34 and ATCC Deposit No. 2031611.

Pending Claims 14-17 are analogous to the claims discussed in Example 14 of the written description training materials available on the PTO’s website. In Example 14, the written description requirement was found to be satisfied for claims directed to polypeptides with 95% homology to a disclosed sequence that also possess a recited catalytic activity, where procedures for making variant proteins were routine in the art and the specification provided an assay for detecting the recited catalytic activity of the protein. This disclosure satisfies the written description requirement even though the applicant had disclosed only a single species and had not made any variants. The Guidelines state that “[t]he single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specified catalytic activity.” (Emphasis added).

Like Example 14, Claims 14-17 have very high sequence homology to the disclosed sequence and must share an epitope sufficient to generate antibodies which specifically detect the polypeptide of SEQ ID NO:34 in esophageal and skin tissue samples. As in Example 14, at the time of the effective filing date of the instant application, it was well known in the art how to make polypeptides with at least 95% amino acid sequence identity to the disclosed sequences.



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*See, e.g., Specification* at ¶¶ [0256]-[0271]. In addition, the specification discloses in detail how to make antibodies which specifically detect a particular PRO polypeptide, and how to use them to detect the PRO polypeptide in a particular tissue. *See, e.g., Specification* ¶¶ [0363]-[0379], [0407], and [0493]-[0499]. Like a particular catalytic activity, the function of being useful to produce an antibody specific to SEQ ID NO:34 is directly related to the structure of the claimed polypeptides. Thus, like Example 14, the genus of polypeptides that have at least 95% amino acid sequence identity to the disclosed sequences and possess the described functional activity are adequately described.

In response to these arguments, the PTO argues that the instant application is not analogous to Example 14 because in Example 14

[M]ethods of generating variants of the protein that have 95% identity and retain its activity are conventional in the art because deletions, substitutions, insertions, and additions of uncritical amino acid residues would not affect the enzyme activity. Moreover, such an enzyme would have a conserved structure that is responsible for the enzyme activity. ... Furthermore, the specification and the claims do not disclose the identification of any particular portion of the PRO1277 structure that must be conserved in order to conserve the required function. *Final Office Action* at 20.

This response is not persuasive, as it mischaracterizes Example 14. Nowhere in Example 14 does it teach that the enzyme is known to have conserved structures that are responsible for the enzyme activity, or that if such structures exist, they were known to those of skill in the art. Instead, the explanation given by the Guidelines for why the specification was adequate to describe the genus is that “[t]he single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound **and** because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specified catalytic activity.” (Emphasis added). Thus, only two things are necessary to provide adequate written description: 1) 95% structural identity to the reference compound, and 2) and assay to identify the variants that have the specified function. Nowhere in the Guidelines is there a discussion of conserved structures that are responsible for the enzyme activity or disclosure of a particular portion of the protein that must be conserved – all that is required at least 95% homology to the disclosed structure and an assay to test for the related function. Applicants have supplied both.

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As for the PTO's conclusory and unsupported statement that "even a very skilled artisan could not envision the detailed chemical structure of all or a significant number of encompassed PRO1277 polypeptides, and therefore, would not know how to make and use them," the basic premise that a large genus can not be adequately described by a single species is simply wrong. In a recent Federal Circuit decision, *In re Wallach*, 378 F.3d 1330, 1333-34 (Fed. Cir. 2004), the Court stated:

[W]e agree with Appellants that the state of the art has developed such that the complete amino acid sequence of a protein may put one in possession of the genus of DNA sequences encoding it, and that one of ordinary skill in the art at the time the '129 application was filed may have therefore been in possession of the entire genus of DNA sequences that can encode the disclosed partial protein sequence, even if individual species within that genus might not have been described or rendered obvious. ... A claim to the genus of DNA molecules complementary to the RNA having the sequences encompassed by that formula, even if defined only in terms of the protein sequence that the DNA molecules encode, while containing a large number of species, is definite in scope and provides the public notice required of patent applicants.

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Moreover, we see no reason to require a patent applicant to list every possible permutation of the nucleic acid sequences that can encode a particular protein for which the amino acid sequence is disclosed, given the fact that it is, as explained above, a routine matter to convert back and forth between an amino acid sequence and the sequences of the nucleic acid molecules that can encode it. *Id.* (emphasis added).

The Court did not require the applicants in *Wallach* to actually make or individually describe all of the vast number of sequences which encode the disclosed sequence. This is in spite of the fact that only a single sequence was disclosed, and the encompassed genus was enormous due to codon degeneracy in the genetic code – even the most skilled artisan could not individually envision the detailed chemical structure of the nucleic acids encompassed by the claimed genus. The Court reasoned that because it is routine to convert between amino acid sequences to nucleic acid sequences, disclosure of a single amino acid sequence was sufficient to place the applicants in possession of the enormous genus of nucleic acids which could encode the sequence. While the Court did affirm the Board's rejection on the grounds that the applicant had sequenced only a portion of a larger protein which it was trying to claim, the Court clearly

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rejected the notion that adequate written description requires that a skilled artisan be able to “envision” the chemical structure of the claimed genus.

The facts in *Wallach* are very similar to the instant case. Here, Applicants have disclosed SEQ ID NO:34, and claim polypeptides which are at least 95% identical to it and have the functional limitation of the ability to generate antibodies which can be used to specifically detect SEQ ID NO:34 in skin tissue samples. As discussed above, it is routine in the art to create polypeptides which have at least 95% sequence identity to SEQ ID NO:34 – it is just as predictable and easy as creating all of the nucleic acids which encode a particular amino acid sequence. Similarly, it is well within the knowledge of those skilled in the art how to determine which polypeptides can be used to make the recited antibodies. The predictability of this structure/function combination is sufficient to place the claimed subject matter in the possession of the Applicants, and thus the claimed polypeptides are adequately described. The *Wallach* opinion makes clear that there is no need to literally describe more than a single species to adequately describe a large genus where one of skill in the art recognizes that the disclosed species puts the applicant in possession of the claimed genus.

As to the PTO’s arguments in the first Office Action that the claims “do not require that the claimed polypeptide possess any particular biological activity, nor any particular conserved structure, or other disclosed distinguishing feature,” these arguments are moot in light of Claims 14-17 which were added after the first Office Action and require a conserved structure as detailed above. Likewise, the PTO’s arguments in the most recent Office Action are directed solely to the amendments to Claims 4 and 5, and do not apply to Claims 14-17.

In conclusion, Applicants submit that they have satisfied the written description requirement for the pending claims based on the actual reduction to practice of SEQ ID NO:34, by specifying a high level of amino acid sequence identity, and by describing how to make antibodies to the disclosed sequence, all of which result in a lack of substantial variability in the species falling within the scope of the instant claims. Applicants submit that this disclosure would allow one of skill in the art to “recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus.” Hence, Applicants respectfully request that the PTO reconsider and withdraw the written description rejection under 35 U.S.C. §112.

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### CONCLUSION

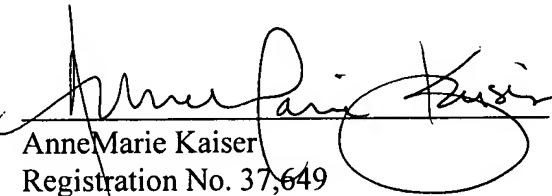
In view of the above, Applicants respectfully maintain that claims are patentable and request that they be passed to issue. Applicants invite the Examiner to call the undersigned if any remaining issues may be resolved by telephone.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

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# Antibodies That React with Predetermined Sites on Proteins

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Progress in molecular biology and virology has always relied heavily on structural analyses of the components of biological systems. Because of advances in nucleic acid biochemistry during the last decade, culminating with the development of rapid DNA sequencing methods that allow the primary chemical sequences of genes to be read (1), we can now often approach a biological problem by identifying and sequencing the responsible genes rather than analyzing the

corresponding to a putative protein deduced from a string of nucleotides. One solution was to make a bacterium (or yeast) synthesize the novel protein and then raise antibodies to the bacterially synthesized protein. However, experience showed that although it was possible to make bacteria synthesize specific proteins if the genes for these proteins had been isolated, each new gene had to be handled individually and required many manipulations (2). If one were in-

**Summary.** Contrary to previous predictions, relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. Peptides capable of eliciting protein-reactive serums are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins nor to the amino or carboxyl terminals. As such, synthetic peptide immunogens are valuable for eliciting reagents with predetermined specificity that can be used for basic research. In addition, some synthetic peptides are capable of mimicking regions of virus proteins and eliciting immune responses in animals that are protective against the viral agents. Such peptides may thus serve as the basis for safe, chemically defined synthetic vaccines.

relevant proteins. In fact, nucleotide sequence analysis of a new virus or gene is usually a most rewarding initial step, since it provides the information the virus or gene uses to accomplish its phenotype as well as a record of the evolutionary history of that nucleic acid. No fact is more important about a gene than its primary sequence; however, proteins are responsible for the execution of most biological processes.

Given the ease with which it was possible to generate primary sequence data for genes, what was needed was a way to link gene sequences to proteins, particularly when a protein was known only by the sequence of a gene. The obvious answer was to use an antibody, but the problem was how to make an antigen

investigating a "gene" whose product was purely hypothetical, a laborious set of experiments might be necessary to achieve its bacterial expression.

An alternative to biological synthesis of antigens is chemical synthesis. However, most biologically interesting proteins whose sequences can be inferred from genes are in the 15,000 to 150,000 dalton range (15K to 150K), whereas chemical synthesis has practical limits in the 4K to 5K range except in exceptional circumstances. Furthermore, most studies of protein immunogenicity (including the work of Landsteiner, Crumpton, Benjamini *et al.*, Atassi and Suplin, Arnon and Sela and their colleagues, and Cebra and others) (3) indicated that small portions of a protein would, in general, be unlikely to elicit antisera reactive against an intact protein [for a recent review, see (4)]. These studies predicted

that immunogenic sites in small, intact proteins occurred about once every 5K to 10K and that these few loci relied on complex tertiary interactions between amino acid residues near each other in the protein tertiary structure, but distant in relation to the primary linear amino acid structure (so-called conformational determinants) (see Fig. 1). Therefore, it was generally believed that linear sequences in short peptides would not usually mimic these important sites. Recent experiments have challenged this prediction and have shown that small, chemically synthesized fragments of a protein can, in fact, elicit antibodies reactive with the native protein, thus allowing nucleic acid sequences to be parlayed quickly into biological experiments.

## Antibodies to Synthetic Peptides React with Native Proteins

The belief that most antigenic determinants are conformational was first challenged in experiments with chemically synthesized protein fragments from the amino or carboxyl terminals of viral proteins whose sequences had been determined from nucleic acid studies. Antibodies to synthetic peptides [prepared as described in (5, 6)] corresponding to the COOH-terminus of the envelope polypeptide of Moloney murine leukemia virus (MuLV) and the NH<sub>2</sub>- and COOH-terminals of the simian virus 40 (SV40) transforming protein were found to be reactive with the native protein structures (7). That is, each reagent was able to precipitate the corresponding protein from extracts of virus-infected cells. In addition, the fact that antibodies to peptides are specific for predetermined sequences within the intact protein permitted analysis of the precursor of the MuLV envelope polypeptide that undergoes two stages of proteolytic cleavage necessary to generate mature viral proteins (8).

The first reports that protein-reactive antibodies could be elicited by synthetic peptides corresponding to fragments of proteins whose sequences were known only from nucleic acid studies (7) gave a clear indication that a powerful technology was now available (9). The possibility remained, however, that the utility of the technique would be confined to the terminals of proteins where carrier-coupled peptides might mimic their position in the intact molecule. We needed to know whether the technique would be applicable to all proteins and if it would be necessary to predict the naturally anti-

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genic regions of proteins in order to select suitable synthetic peptides (10). We used the recently derived sequences of the hepatitis B virus surface antigen (HB<sub>s</sub>Ag) and the hemagglutinin gene of influenza virus type A (HA1) (11) to examine the general utility and expand our understanding of this technology. The two sequences offered different experimental situations. The HB<sub>s</sub>Ag was known to be a molecule whose immunogenicity is critically dependent on its native tertiary structure (12); furthermore, it is extremely hydrophobic. The crystallographic structure of influenza A hemagglutinin had recently been solved (13) and its dominant antigenic sites were well known (14). In addition, since both molecules are the primary targets of neutralizing antibody during natural infection they are of practical interest in dealing with human disease (14-16).

One question we needed to answer concerned the peptide characteristics required to elicit antibodies reactive with the native molecule: Are there restrictions in the chemical makeup or location within the protein structure? The hydrophobic HB<sub>s</sub>Ag glycoprotein consists of 226 amino acid residues. We synthesized 13 peptides using the HB<sub>s</sub>Ag amino acid sequence deduced from the hepatitis B genome sequence as a blueprint (17). These peptides were distributed throughout the primary sequence, but avoided in those regions that showed significant variability in the different viral isolates. Four were subsets of longer peptides. From the data obtained with these peptides we were able to formulate an elementary set of rules for selecting peptides capable of eliciting antisera reactive with native proteins. Peptides that were extremely hydrophobic and those of six or fewer residues were ineffective; longer, soluble peptides, especially those containing proline residues, were effective. Antisera against four of the six HB<sub>s</sub>Ag peptides in the latter category precipitated the HB<sub>s</sub>Ag protein in the viral Dane particles. Precipitation also occurred under conditions approximating physiological (saline solutions), indicating that antibodies to these peptides might be expected to bind antigen *in vivo*. This study showed that linear peptides from more than one region of a protein and, more important, not restricted to its NH<sub>2</sub>- or COOH-terminus could elicit protein-reactive antibodies.

We also needed to know the relation between the sites represented by effective peptides and the antigenic determinants selected by the host in the course of a natural immune response against a virus or a protein. For influenza virus,

those sites immunogenic during natural infection had been mapped by analysis of variants to four domains of the HA1 chain of the hemagglutinin molecule, whose three-dimensional structure was known from x-ray crystallographic studies (13-15). We studied 20 synthetic peptides, many of them overlapping, covering 75 percent of the HA1 primary sequence derived from the nucleotide sequence of a fragment of the influenza genome (6). In accordance with the rules formulated in the HB<sub>s</sub>Ag study (17), these peptides ranged in length from 8 to 39 residues, contained enough polar amino acids to render them soluble and, hence, easy to work with, and usually contained one or more proline residues. Some of the peptides fell within the known antigenic domains of HA1; others were clearly outside these domains. The peptides correspond to regions of the

protein which in the crystal structure appeared as  $\alpha$ -helices,  $\beta$ -sheets, and random coils. Antibodies to 18 of the 20 peptides reacted with HA1 (isolated by bromelain cleavage) or intact virus, demonstrating that sites in proteins accessible to peptide antibodies are more numerous than the few sites recognized in the course of a natural immune response. Furthermore, the information carried within a relatively short linear peptide is sufficient to elicit reactivity against a much larger protein molecule with a complex tertiary and quaternary structure. In more recent studies with peptides selected by the same rules outlined above, 12 of 12 peptides predicted from the MuLV polymerase gene (18) and 18 of 18 peptides from the rabies glycoprotein gene (19) elicited antibodies that precipitated their putative corresponding proteins. Therefore, it appears that, by

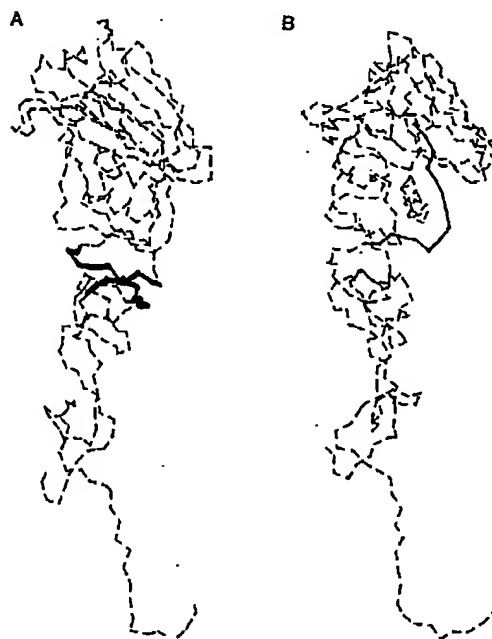


Fig. 1. Simplistic diagrams depicting (A) conformational and (B) sequential (or linear) determinants. The atomic coordinates of the influenza A/Hong Kong HA1 crystallographic structure (13) were rotated until prototypical regions recognized by eye were portrayed with Evans-Sutherland graphics by means of the GRAMPS and GRANNY programs of Donnell and Olson (59) and Connolly (60) to emphasize specific molecular features. The two panels show HA1 at different rotations around its vertical axis. The relevant regions are shown by the solid lines (in bold face in (A)); the rest of the molecule is shown by dashed lines along the  $\alpha$ -carbon backbone. (A) The molecule shows a conformational determinant—two regions of the HA1 polypeptide chain (residues 50 to 55 and 270 to 280) are far apart in the linear protein sequence but intimately related with each other through a disulfide linkage.

The chains from the two regions track each other for several residues. One might expect that an antibody response to this region would probably involve contacts with both peptide chains. This appears to be the case during natural infection; this is HA1 site C of (14). However, antisera to synthetic peptides representing either chain are capable of precipitating HA1 and, in fact, neutralizing the influenza virus. (B) The molecule shows a sequential determinant. The conformation of the peptide chain (residues 255 to 268) in the region shown by the solid line is mostly determined by the particular residues of the chain itself because no other close intramolecular contacts are apparent (nor are there any contacts with HA2 or other HA1 + 2 protomers in the trimeric form of the hemagglutinin). A synthetic peptide might well be expected to antigenically simulate a region such as this. These two extremes are simplifications that help to clarify abstract discussions of protein structure and immunogenicity. In reality, lengthy sequential regions of a protein usually have somewhat extensive contacts with other regions of the protein. However, linear peptides can often mimic the intact protein structure by eliciting a protein-reactive antiserum; therefore, some of the forces that mold global protein structure are already contained in relatively short peptide regions. Clearly, antibody molecules react with a number of spatially related atoms in the antigen molecule, and, in that sense, all determinants are conformational. Recent observations indicate that many peptides from within a protein molecule can act as antigenic determinants; thus many determinants are also sequential. The conformational-sequential nomenclature is probably no longer a useful one.

following simple rules, one can in general select peptides that will elicit antibodies reactive with intact proteins.

In retrospect, it is easy to understand why, in terms of their chemistry, synthetic peptides have been so successful as immunogens in these and several other recent studies and why it was previously believed that they would be ineffective and were therefore not vigorously studied. All of the effective peptides from the influenza HA1 study (6) could be shown, when matched to the crystallographic structure of the protein, to represent regions exposed to the solvent. This may be due, in part, to the fact that they contain polar residues. Polar residues may also provide components of antigenic determinants capable of forming strong electrostatic interactions with antibody. In addition, proline residues may be important because they occur at bends in the peptide chain, often at "corners" exposed to the solution. Because proline residues have an imide rather than an amide bond, several atoms surrounding a proline residue have a fixed three-dimensional relation to one another, whether in a short peptide or in a complete protein structure, and, as such, will be recognized equally by antibody in either situation. Furthermore, residues on the two sides of a proline tend to be near each other because the peptide chain more or less turns back on itself at *cis* proline kinks. This produces a two-chain structure which might be thought of as a minor "conformational" determinant, albeit formed by residues not too far from one another in the linear chemical formula of the protein. Although these explanations for the effectiveness of peptides with certain chemical properties are somewhat speculative, it is now clear that, in solution, peptides and proteins are often similar. Peptides in solution probably attain conformations dictated by their chemical makeup which resemble those that occur in native protein structures. Furthermore, proteins are probably not static structures, always closely resembling their crystalline form; in fact, they may exhibit their various linear domains in many conformations. In support of these notions, Niman and Lerner (20) have shown that 50 percent of the monoclonal antibodies selected for reactivity with one of the peptides in the influenza HA1 study also react with HA1 protein.

In contrast, antisera raised against native HA1 do not react with any of the 20 HA1 peptides (6). Since these peptides span 75 percent of the HA1 primary sequence, including all of the known, mapped determinants, it is clear that

most, if not all, of the immune response against native HA1 is directed against determinants not mimicked by short linear peptides. This observation is consistent with the body of data showing that antigenic determinants on intact proteins are largely conformational (3). Earlier investigators reasoned that because the determinants were conformational, only rarely would a linear peptide be an effective antigen; therefore synthetic peptides would not have general utility. The influenza HA1 study shows that pieces of a protein can elicit antibodies reactive with the whole protein which the whole protein itself cannot elicit (6). Moreover, the studies on HB<sub>s</sub>Ag, influenza HA1, MuLV polymerase, and rabies glycoprotein demonstrate that these peptides are not difficult to select (6, 17-19). On the basis of the rules formulated in the HB<sub>s</sub>Ag study, one can select one or two peptides from a protein sequence with relatively high confidence of being able to elicit a protein-reactive antiserum, an important economic consideration both in time and research dollars.

#### Protein-Reactive Peptide Antibodies as Reagents for Molecular Studies

Synthetic peptides that elicit reagents capable of reacting with proteins of known primary sequence can be used to establish identity between a protein sequence and the protein itself. Many sequences are of a hypothetical nature, having been deduced from a gene sequence, and an experiment with a synthetic peptide may be more powerful for demonstrating colinearity between a protein and nucleic acid than a series of successful genetic experiments. Antisera to peptides have been used to detect the putative products of the MuLV polymerase (18) and envelope (7) genes as well as the transforming genes of the Moloney sarcoma (21), simian sarcoma (22), feline sarcoma (23), avian myeloblastosis (24), SV40 (7), and polyoma (25) viruses. Such antisera have also been used in similar studies of six human adenovirus 2 (26) transforming genes, the mouse mammary tumor virus long terminal repeat (LTR) (27), and several messenger RNA's from rat brain (28). Peptides from the NH<sub>2</sub>- and COOH-terminals as well as from the middle of the adenovirus transforming proteins elicit protein-reactive sera. Antibodies to peptides have been used to track the processing of polypeptide precursors in cells infected with poliovirus (29), influenza (Fig. 2), and MuLV (8, 18). Because of the predetermined specificity

of the antisera elicited by synthetic peptides, the proteins reacting with them are known to carry the specific peptide sequence. These reagents are particularly powerful when used as sets for tracking simultaneously the fate of various regions of a protein precursor (as shown for influenza HA in Fig. 2 and discussed below for the leukemia virus *pol* product). They are also useful in identifying alternative exon usage [as has been shown with immunoglobulin class D genes (30) and the adenovirus 2 E1A transcription unit (31)]. With the adenovirus 2 E1B transcription unit synthetic peptides have been used to show that the 53K and 19K protein products are translated in different triplet reading frames (26).

When the existence of a protein has been demonstrated, one wants to know its cellular location. Antisera to a peptide from the COOH-terminus of the vesicular stomatitis virus (VSV) G protein do not react with intact infected cells, although polyclonal antisera to the G protein do. When the cells are opened by treatment with detergent, a strong reaction at the inner surface of the membrane is detected with the antiserum to the COOH-terminal peptide (32). These experiments indicate that the VSV G protein spans the plasma membrane with its COOH-terminus protruding into the cytoplasm. In immunofluorescence studies of fixed, transformed fibroblast cells, antisera to a COOH-terminal peptide from the Rous sarcoma virus (RSV) transforming protein were used (33). Fluorescence was codistributed with vinculin at cell-cell contact sites. Antiserum to an interior RSV *src* peptide [which cross-reacts with the endogenous cellular *src* as well as the transforming proteins of Fuginami (*fps*) and Y73 (*yes*) viruses] reacted with the focal adhesion plaques of RSV-transformed rat cells (34). Therefore, these reagents, derived by using the sequence of a protein (or gene), are capable of indicating in detail the cellular location of a protein.

Another use for peptide antisera is the correlation of structure with function. If an antiserum to a peptide perturbs an assayable protein function, a protein containing the peptide sequence is implicated. Antiserum to a peptide present in the middle T sequence of polyoma virus inhibits protein kinase function *in vitro* (35), and an antiserum to a peptide present in the feline sarcoma virus *fes* gene sequence also inhibits protein kinase activity (23). Therefore, each of these proteins must have protein kinase activity: the predetermined specificity of the reagent allows one to rule



out the often cited caveats about proteins and enzymatic activities that happen to coincide. Antisera against several peptides in the NH<sub>2</sub>-terminal half of the putative MuLV *pol* polyprotein inhibit reverse transcriptase activity, whereas some peptides in the COOH-terminal portion inhibit a virion-associated endonuclease activity (18). The two sets of inhibiting antisera immunoprecipitate two different proteins in infected cells, as well as their common precursor. Therefore, not only do the peptide antisera establish the colinearity between the *pol* protein precursor and its nucleic acid sequence, but they identify the products of protein maturation and assign them an enzymatic activity. Antiserum to a COOH-terminal peptide predicted from the poliovirus replicase gene sequence precipitates the core protein p63 and its precursors and inhibits replicase and polyuridylic acid polymerase activities *in vitro*, indicating that these two activities reside in p63 (36). One can thus imagine a range of experiments in which peptide antisera will be used to modify specific behaviors of proteins because they bind to a specific predetermined region of that protein.

When neither function nor structure is known, antisera to peptides can provide a means for purifying the bona fide protein. Walter and his colleagues (37) have coupled to Sepharose the purified immunoglobulin fraction of a peptide antiserum and used this to purify the middle T antigen of polyoma virus. The protein was eluted from the immunoaffinity column by competition with excess peptide. A two-cycle purification scheme in which one uses the immunoglobulin fractions of antisera against two different peptides from a protein sequence might lead to a quite pure protein preparation (37). Immunoprecipitation and gel electrophoresis have been used to isolate a precursor of the MuLV envelope membrane anchor to a purity such that its NH<sub>2</sub>-terminal sequence could be determined by radiochemical sequencing methods (8). It might eventually be possible to use the complement fixation properties of antigen-antibody complexes as an assay for protein purification after various chromatographic steps (38).

Although most work with synthetic peptides has been done with antisera raised in laboratory animals, some investigators have used clonal populations of peptide-specific lymphocytes for their experiments. The studies of Niman and Lerner (20) on influenza HA1, and those of Gentry *et al.* (34) on the RSV transforming protein, have shown that a high

proportion of hybridomas obtained by fusion of spleen cells from peptide-immunized donors secrete antibody reactive with the native protein. Such doubly specific reagents can be prepared in large quantities and are particularly useful for studies of fixed cells or tissues or for large-scale diagnostic work. Monoclonal populations of B-cell precursors, which can bind peptide but are not yet capable of antibody secretion, have been used to probe the immunological repertoire to determine whether antigen tolerance to host proteins is inherited or acquired (39). In the influenza system, Lamb and his co-workers (40) found that hemagglutinin-specific T-cell populations from human donors responded to 12 of 12 HA1 peptides analyzed. One peptide, corresponding to the COOH-terminal 24 amino acids, appeared to be immunodominant in the sense that three of four T-cell clones were specific for determinants within this sequence.

On the technical side, antibody titer is a relevant concern. Some peptides seem

highly immunogenic, sometimes even without being coupled to carrier protein, eliciting antisera of high titer after a 35-day immunization procedure in which three doses, each of 200 micrograms of peptide, are administered (5, 6). Other peptides seem to require several months and several booster injections to achieve a reasonable titer. A second immunization procedure in which the peptide is coupled to a carrier protein different from that used in the initial series of inoculations seems to be very effective for eliciting a peptide-specific rather than a carrier-specific response (28). For many, but not all, peptides we have examined, glutaraldehyde coupling seems to be more effective than coupling through cysteine residues. However, glutaraldehyde modifies lysine NH<sub>2</sub> groups and hence can significantly interfere with key amino acids in some peptides. To increase the activity of antisera, and to reduce background activities, purified immunoglobulin can be specifically enriched by adsorption to the antigenic peptide coupled to a solid support (25). The immunoglobulin is then released by a chaotropic agent or removed from the column by competition with excess peptide, then dialyzed in the presence of a chaotropic agent.

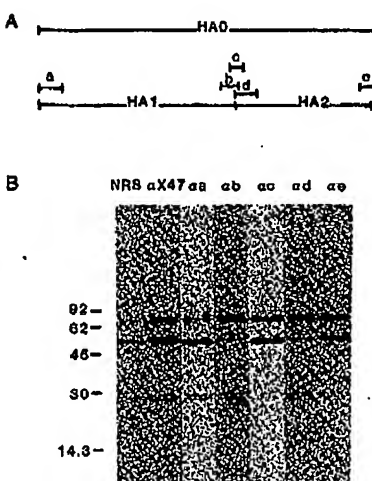


Fig. 2. Illustration of the power of sets of peptide antisera used to track distinct regions of a protein simultaneously. (A) The influenza hemagglutinin precursor (HA0) and eventual products (HA1 and HA2). The synthetic peptides a to e, located within the HA0 sequence as shown (NH<sub>2</sub>- and COOH-terminal, flanking and spanning the HA1-HA2 junction), were coupled to a carrier and used to immunize rabbits as described (5-7). (B) The resulting antisera (aa, ab, ac, ad, ae) and a positive control serum (antibody to X47 influenza virus, αX47) and a negative control serum (normal rabbit serum, NRS) were used to precipitate extracts of [<sup>35</sup>S]methionine-labeled influenza X47 virus-infected cells. The five antisera to the peptides and αX47 precipitated the HA0 molecule (this precursor is not appreciably cleaved during infection of cell lines), whereas the other precipitated proteins were nonspecific (sticky) since they also appeared in the normal control lane.

## Synthetic Peptides as Ideal Vaccines

For our studies we have used the immunological targets of infectious pathogens with the goal of eventually applying our findings to the protection of humans and other animals from disease by vaccination with synthetic peptides. Indeed, previous workers have shown that peptides of natural or synthetic origin can elicit antisera capable of neutralizing or binding to virus or bacteriophage *in vitro* [for a review, see (41)]. The coat proteins of tobacco mosaic virus (TMV) (42) and more recently bacteriophage MS2 (43) were fragmented with proteases, and fragments that reacted with neutralizing sera to whole virus were identified and isolated biochemically or chemically synthesized. Antiserum to the natural COOH-terminal six residues of TMV had the ability to bind to the virus *in vitro* and abrogate its infectivity. Antisera to a synthetic MS2 peptide reduced this bacteriophage's infectivity when a secondary antibody to the antibody or "sandwich" reaction was used (no primary neutralization was demonstrated). Similarly, Audibert *et al.* (44) demonstrated that antisera against a synthetic peptide from the diphtheria toxin was capable of



inhibiting toxin activity in vitro. Our studies on HB<sub>s</sub>Ag (17) and influenza HA1 (6) showed that it is possible to select, on the basis of simple chemical properties of regions within viral proteins, peptide immunogens that induce virus-specific antibody. However, these studies did not establish the relation between the capacity to bind virus and the capacity to neutralize infection. What was needed was a demonstration of active protection from a virulent disease in an immunized animal.

In terms of neutralization, there seem to be two classes of virus—those that can be neutralized by antiserum to related viruses and those that are quite subtype-specific in their cross-reactivity. Protective peptides from the first class might cluster in regions whose sequences are conserved across related viruses, whereas protective peptides from the second class may reside in the variable regions. Although it is not necessarily the case that the serum of peptide-protected animals will mimic that of animals protected by classical vaccines, we have proceeded with the thought that reproducing the known serology may be important. The most extensive studies to date have been with four viruses: foot-and-mouth, influenza A, hepatitis B, and rabies.

Seven peptides were chosen (45) from the translated nucleic acid sequence of the type O foot-and-mouth disease virus (FMDV) VP1 protein (46). The VP1 was thought to be a target for neutralizing antibodies, because treatment of this picornavirus with trypsin cleaves only VP1 and this is sufficient to cause the virus particle to lose its immunizing activity (47). Rabbits inoculated with each of the seven peptides made strong antipeptide responses, but antisera to only three of the peptides were capable of neutralizing FMDV in vitro (45). The neutralization was specific to type O virus, as expected from serological considerations. In addition, guinea pigs immunized with a single dose of either of two of the effective peptides (in complete Freund's adjuvant or alum) and challenged with live virus were protected. Thus, a synthetic peptide can elicit a protective immune response. Indeed, one of the peptides that was 20 residues long was as good at protecting the animals from later challenge with 10<sup>4</sup> infectious doses (ID<sub>50</sub>) of FMDV as inactivated virus particles, the classical vaccine (45). Antisera raised in rabbits or cattle against peptides from the equivalent region of other FMDV strains neutralize their appropriate strain of virus. These results are particularly encouraging be-

cause VP1 itself, whether purified from virus or made by genetically engineered bacteria, is a poor immunogen which thus far has not lent itself to the production of a subunit vaccine.

The antipeptide serums to influenza HA1 peptides (6) have been assayed for virus neutralization. Antibodies to 6 of the 20 peptides described in our original structural study neutralized the cytopathic effect of influenza virus on MDCK (canine kidney) cells (48). Subsequently, six more peptides were synthesized filling in the regions missed in the original study, including the HA1-HA2 junction and the other chain of the site C of Wiley *et al.* (14). Antisera to five of these six peptides neutralize the cytopathic effect in vitro (48). In addition, immunizations with several combinations of peptides from the HA1 sequence protect mice from death caused by mouse-adapted influenza virus. However, only two of the five peptides thus far injected individually confer active immunity. Müller *et al.* (49) report neutralization of the cytopathic effect with antiserum to one influenza peptide and inhibition of virus growth in vivo by immunization with that peptide. Although it is too soon to make generalizations about structure, some statements about broad-range neutralization as measured in vitro by Alexander *et al.* (48) are possible. Sequences in the site C region (defined by a disulfide bond between residues 52 and 277 in the native HA1) (14), the COOH-terminal region of HA1 and the HA1-HA2 junction are relatively conserved in the several type A influenza hemagglutinins that have been sequenced. Antisera to peptides from each of these regions neutralize not only viruses of the H<sub>3</sub> subtype (from which the sequence was derived) but also H<sub>1</sub> and B viruses (H<sub>2</sub> has not been tested). Normally, antisera against one subtype of influenza virus do not protect against other subtypes. Thus with peptides it may be possible to construct a vaccine with a broad range of specificity not attainable with the intact protein.

The only relevant model for human hepatitis B is the chimpanzee, so direct studies of protection have not been done; however, extensive serological studies have been conducted in vitro. Prince *et al.* (50) demonstrated that a synthetic peptide corresponding to HB<sub>s</sub>Ag residues 138 to 149 inhibited the binding of hybridomas against the a and d but not the y subdeterminants of HB<sub>s</sub>Ag. In apparently conflicting studies carried out in the chimpanzee, Gerin *et al.* (51) report that a peptide spanning the region 110 to 137 contains the a and y but

not the d specificity (d and y are thought to be allelic). This result is consistent with the results of protein chemistry studies of Peterson *et al.* (52), which show the y/d variation to occur at residues 131 and 134. This is supported by our studies showing that a synthetic peptide corresponding to residues 110 to 137 of the d version elicits d-specific antiserum. Bhatnagar *et al.* (53) have recently reported that a synthetic peptide covering the region 139 to 147 carries the a but not the y/d determinant. The reason for the discrepancy between the results of Prince *et al.* (50) and all of the others is not known, but whichever studies are correct, peptides seem to be able to duplicate serologically important antigenic determinants and elicit immune responses in primates corresponding to those seen during infection. No data on neutralization of the virus or active protection against infection are yet available.

In collaboration with colleagues at the Wistar Institute (19), we have investigated the efficacy of synthetic peptides in protecting animals from rabies virus infection. The purified rabies viral glycoprotein is capable of conferring protection from rabies virus, and all neutralizing sera and hybridomas precipitate this protein (54). Eighteen peptides, representing 56 percent of the primary rabies glycoprotein sequence as deduced from the nucleic acid sequence of its gene (55), were originally synthesized and antibodies were raised against these in rabbits and mice (19). All 18 peptides elicited antisera that bound both rabies glycoprotein and virus as measured in ELISA assays. However, none was capable of neutralizing the virus in vitro or of protecting mice or dogs from challenge from live virus. More recently, it has been shown that the three cyanogen bromide fragments of the glycoprotein capable of eliciting protection were not well covered by the peptide selection (56), hence experiments directed at synthesizing these particular regions are under way. So, while it was easy to pick 18 out of 18 peptides that would elicit binding antibodies, none of these seems capable of protecting against the virus.

Clearly, the studies of FMDV and influenza virus show that one can protect an animal from infection with live virus by injecting synthetic peptides corresponding to parts of the proteins that are the normal targets of neutralizing antibodies. However, the studies with rabies and influenza virus demonstrate that binding antibodies, while easy to elicit, are not necessarily neutralizing antibodies. From the medical-veterinary point of view, one must either resort to synthe-

sizing a series of overlapping peptides covering a complete protein sequence (now a technically feasible, if not particularly elegant, approach) or perform supporting biological experiments to determine which regions of a protein contain its neutralization sites. Although sites capable of interacting with antibody are probably located on the entire surface of the viral protein with frequent representation in its primary linear amino acid sequence, and although such sites can probably be easily mimicked by synthetic peptides, sites susceptible to neutralization seem much less frequent and have not yet been characterized by a set of simple chemical properties. Nonetheless, once the neutralization sites are identified, synthetic peptides seem to be suitable substitutes for whole proteins or viruses (and presumably other infectious agents). For some sites, they may be more effective immunogens than whole protein because they can elicit specificities that whole proteins cannot.

If one projects this technology from prophylaxis to immunological therapy, synthetic peptides from proteins unique to tumor cells may be suitable immunogens for treating neoplasia. The peptides may be from proteins unique to the clonal population of transformed cells or, in tumors of tissues that are dispensable (such as prostate or thyroid), the peptides may be merely tissue-specific, cell-surface markers.

## Conclusions

The desirability of synthetic vaccines of known potency and side effects has been recognized for many years. Some of the drawbacks of currently available inactivated virus vaccines are that they are not stable without refrigeration and sometimes contain incompletely inactivated viruses capable of starting minor epidemics (57). Vaccination with a substance free of any biological contamination introduced in its production (either by virus grown in cell culture or in genetically engineered proteins expressed by *Escherichia coli* protein synthesis factories) and unable to cause any virus-related pathology because of incomplete virus inactivation or imperfect attenuation is the logical goal of protection from infectious disease.

Certainly this technology has not been developed to the level of sophistication that is required for the widespread use of synthetic vaccines in humans and other animals. The adjuvants and carriers used in the studies described in this article are in general much too harsh for human

use, although alum (used in the FMDV study) is suitable. The work of Chedid, Audibert, and Langbeheim and their colleagues (58) may indicate a possible direction for more suitable and ultimately totally defined vaccines. Suitable doses, the possibility of using modified peptides or combinations or polymers of peptides, and the various routes of injection need to be worked out. But a major theoretical obstacle has been overcome in that solving and then synthesizing complex conformational determinants no longer seems necessary.

Now that it has been shown that protection by synthetic peptides is possible, that such peptides can be at least as effective as biological vaccines, that new protein sequences are rapidly being generated as a result of nucleic acid studies, and that the synthetic approach is economically quite feasible (whether it requires the synthesis, by brute force, of all fragments of a protein or directed biochemical-immunological experiments), research to find the best peptides, the best adjuvants, and the best carriers is likely to become an important priority. As the research progresses, and as synthetic vaccines become commonplace, the somewhat tedious processes used today will become streamlined and consequently much more economical.

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## Protein Engineering

Kevin M. Ulmer

In the last decade, genetic engineering technology has been developed to the point where we can now clone the gene for essentially any protein found in nature. By precise manipulation of the appropriate regulatory signals we can then produce significant quantities of that protein in bacteria. Recent advances in chemical synthesis of DNA now permit virtually unlimited genetic modification, and offer the prospect for developing protein engineering technology to create novel proteins not found in nature. By starting with the known crystal structure for a protein we would like to directly modify the gene to alter that structure in a predictable fashion, targeted to improve some functional property. At each stage we could verify the structural and functional changes that actually occurred and thereby refine and extend our predictive capability. Step by step, as we gain facility with this technique and learn the detailed rules that relate structure and function, we should be able to create proteins with novel properties which could not be achieved as effectively by any other method.

### Rationale

Despite the fact that biochemists have characterized several thousand enzymes, there are only a handful that could be considered enzymes of commerce. Indeed, only a dozen enzymes have worldwide sales in excess of \$10 million per year, and together they ac-

count for more than 90 percent of the total enzyme market (1). Frequently the limiting factor in the industrial use of an enzyme has simply been the high cost of isolating and purifying adequate amounts of the protein. Part of the solution to this problem lies with the ability of genetic engineers to greatly amplify the produc-

**Summary.** The prospects for protein engineering, including the roles of x-ray crystallography, chemical synthesis of DNA, and computer modeling of protein structure and folding, are discussed. It is now possible to attempt to modify many different properties of proteins by combining information on crystal structure and protein chemistry with artificial gene synthesis. Such techniques offer the potential for altering protein structure and function in ways not possible by any other method.

tion of specific enzymes in microorganisms, but beyond cost there are often other limitations to the broader use of enzymes which stem from the fact that the desired industrial application is far removed from the physiological role normally played by the enzyme. In particular, industrial applications require generally robust enzymes with a long half-life under process conditions. Frequently the desired substrate or product is somewhat different from the physiological one, and often the chemical conditions for the reaction are decidedly nonphysiological, ranging to extremes of pH, temperature, and concentration. If enzymes are to be more widely used as industrial catalysts, we must develop methods to tailor their properties to the process of interest. The list of properties of enzymes we would like to be able to con-

trol in a predictable fashion would include the following:

- 1) Kinetic properties including the turnover number of the enzyme and the Michaelis constant,  $K_m$ , for a particular substrate.
- 2) Thermostability and temperature optimum.
- 3) Stability and activity in nonaqueous solvents.
- 4) Substrate and reaction specificity.
- 5) Cofactor requirements.
- 6) pH optimum.
- 7) Protease resistance.
- 8) Allosteric regulation.
- 9) Molecular weight and subunit structure.

The solutions to these problems have included extensive searches for the best suited naturally occurring enzyme, mu-

tation and selection programs to enhance the native enzyme's properties, and chemical modification and immobilization to obtain a stable and functional biocatalyst. From such work we know that all of these properties can in general be improved. Specific examples of what has been achieved by these methods and how protein engineering can build on this knowledge to yield still further improvements are cited below.

It is not uncommon to observe wide variations in properties such as turnover number,  $K_m$ , molecular weight, temperature optimum, thermostability, pH optimum, and pH stability among enzymes of the same type isolated from different

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## **Modulation of the glutamatergic receptors (AMPA and NMDA) and of glutamate vesicular transporter 2 in the rat facial nucleus after axotomy.**

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Facial nerve axotomy is a good model for studying neuronal plasticity and regeneration in the peripheral nervous system. We investigated in the rat the effect of axotomy on the different subunits of excitatory glutamatergic AMPA (GLuR1-4), NMDA (NR1, NR2A-D) receptors, post-synaptic density 95, vesicular glutamate transporter 2, beta catenin and cadherin. mRNA levels and/or protein production were analyzed 1, 3, 8, 30 and 60 days after facial nerve axotomy by in situ hybridization and immunohistofluorescence. mRNAs coding for the GLuR2-4, NR1, NR2A, B, D subunits of glutamatergic receptors and for post-synaptic density 95, were less abundant after axotomy. The decrease began as early as 1 or 3 days after axotomy; the mRNAs levels were lowest 8 days post-lesion, and returned to normal or near normal 60 days after the lesion. The NR2C subunit mRNAs were not detected in either lesioned or intact facial nuclei.

Immunohistochemistry using specific antibodies against GLuR2-3 subunits and against NR1 confirmed this down-regulation. There was also a large decrease in vesicular glutamate transporter 2 immunostaining in the axotomized facial nuclei at early stages following facial nerve section. In contrast, no decrease of NR2A subunit and of post-synaptic density 95 could be detected at any time following the lesion. beta Catenin and cadherin immunoreactivity pattern changed around the cell body of facial motoneuron by day 3 after axotomy, and then, tends to recover at day post-lesion 60 days. Therefore, our results suggest a high correlation between restoration of nerve/muscle synaptic contact, synaptic structure and function in facial nuclei. To investigate the mechanisms involved in the change of expression of these proteins following axotomy, the facial nerve was perfused with tetrodotoxin for 8 days. The blockade of action potential significantly decreased GLuR2-3, NR1 and NR2A mRNAs in the ipsilateral facial nuclei. Thus, axotomy-induced changes in mRNA abundance seemed to depend partly on disruption of activity.

PMID: 16182453 [PubMed - in process]

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**The alpha(v)beta6 integrin receptor for Foot-and-mouth disease virus is expressed constitutively on the epithelial cells targeted in cattle.**

**Monaghan P, Gold S, Simpson J, Zhang Z, Weinreb PH, Violette SM, Alexandersen S, Jackson T.**

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Field strains of Foot-and-mouth disease virus (FMDV) use a number of alpha(v)-integrins as receptors to initiate infection on cultured cells, and integrins are believed to be the receptors used to target epithelial cells in animals. In this study, immunofluorescence confocal microscopy and real-time RT-PCR were used to investigate expression of two of the integrin receptors of FMDV, alpha(v)beta6 and alpha(v)beta3, within various epithelia targeted by this virus in cattle. These studies show that alpha(v)beta6 is expressed constitutively on the surfaces of epithelial cells at sites where infectious lesions occur during a natural infection, but not at sites where lesions are not normally formed. Expression of alpha(v)beta6 protein at these sites showed a good correlation with the relative abundance of beta6 mRNA. In contrast, alpha(v)beta3 protein was only detected at low levels on the vasculature and not on the epithelial cells of any of the tissues investigated. Together, these data suggest that in cattle, alpha(v)beta6, rather than alpha(v)beta3, serves as the major receptor that determines the tropism of FMDV for the epithelia normally targeted by this virus.

PMID: 16186231 [PubMed - in process]

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## **Cyclin D1-negative mantle cell lymphoma: a clinicopathologic study based on gene expression profiling.**

**Fu K, Weisenburger DD, Greiner TC, Dave S, Wright G, Rosenwald A, Chiorazzi M, Iqbal J, Gesk S, Siebert R, De Jong D, Jaffe ES, Wilson WH, Delabie J, Ott G, Dave BJ, Sanger WG, Smith LM, Rimsza L, Braziel RM, Muller-Hermelink HK, Campo E, Gascoyne RD, Staudt LM, Chan WC; Lymphoma/Leukemia Molecular Profiling Project.**

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Cyclin D1 overexpression is believed to be essential in the pathogenesis of mantle cell lymphoma (MCL). Hence, the existence of cyclin D1-negative MCL has been controversial and difficult to substantiate. Our previous gene expression profiling study identified several cases that lacked cyclin D1 expression, but had a gene expression signature typical of MCL. Herein, we report the clinical, pathologic, and genetic features of 6 cases of cyclin D1-negative MCL. All 6 cases exhibited the characteristic morphologic features and the unique gene expression signature of MCL but lacked the t(11;14)(q13; q32) by fluorescence in situ hybridization (FISH) analysis. The tumor cells also failed to express cyclin D1 protein, but instead expressed either cyclin D2 (2 cases) or cyclin D3 (4 cases). There was good correlation between cyclin D protein expression and the corresponding mRNA expression levels by gene expression analysis. Using interphase FISH, we did not detect chromosomal translocations or amplifications involving CCND2 and CCND3 loci in these cases. Patients with cyclin D1-negative MCL were similar clinically to those with cyclin D1-positive MCL. In conclusion, cases of cyclin D1-negative MCL do exist and are part of the spectrum of MCL. Up-regulation of cyclin D2 or D3 may substitute for cyclin D1 in the pathogenesis of MCL.

PMID: 16123218 [PubMed - indexed for MEDLINE]

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## **BCL2 protein expression parallels its mRNA level in normal and malignant B cells.**

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The regulation of B-cell lymphoma 2 (BCL2) protein expression in germinal center (GC) B cells has been controversial. Previous reports have indicated posttranscriptional regulation plays a dominant role. However, a number of recent studies contradicted these reports. Using real-time polymerase chain reaction (PCR) and Standardized Reverse Transcriptase-PCR (StaRT-PCR), we measured the level of mRNA expression in GC, mantle zone (MNZ), and marginal zone (MGZ) cells from laser capture microdissection. Both quantitative RT-PCR measurements of microdissected GC cells from tonsils showed that GC cells had low expression of BCL2 transcripts commensurate with the low protein expression level. These results are in agreement with microarray studies on fluorescence-activated cell sorter (FACS)-sorted cells and microdissected GC cells. We also examined BCL2 mRNA and protein expression on a series of 30 cases of diffuse large B-cell lymphoma (DLBCL) and found, in general, a good correlation. The results suggested that BCL2 protein expression is regulated at the transcriptional level in normal B cells and in the neoplastic cells in most B-cell lymphoproliferative disorders.

PMID: 15242877 [PubMed - indexed for MEDLINE]



## **Galanin in pituitary adenomas.**

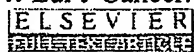
**Grenback E, Bjellerup P, Wallerman E, Lundblad L, Anggard A, Ericson K, Aman K, Landry M, Schmidt WE, Hokfelt T, Hulting AL.**

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Tumor galanin content was measured in extracts from human pituitary adenomas using a specific RIA method for monitoring human galanin. Twenty-two out of twenty-four tumors contained galanin with notably high levels in corticotroph adenomas, varying levels in clinically inactive tumors, and low levels in GH secreting adenomas. Tumor galanin and ACTH contents were closely correlated in all tumors. In four young patients with microadenomas and highly active Mb Cushing tumor galanin was inversely related to tumor volume. The molecular form of tumor galanin, studied with reverse-phase HPLC, was homogeneous with the majority of tumor galanin coeluting with standard human galanin. In the tumors analysed with in situ hybridization there was a good correlation between galanin peptide levels and galanin mRNA expression. In some tumors galanin mRNA and POMC levels coexisted, in others they were essentially in different cell populations. Levels of plasma galanin-LI were not related to tumor galanin concentration, and galanin levels were in the same range in sinus petrosus close to the pituitary venous drainage as in peripheral blood. Corticotrophin releasing hormone injections in two patients caused ACTH, but no detectable galanin release into sinus petrosus. Our results demonstrate that corticotroph, but not GH adenomas, express high levels of galanin, in addition to ACTH, and that in some tumors both polypeptides are synthesised in the same cell population. However, galanin levels in plasma were not influenced by the tumor galanin content.

PMID: 14700749 [PubMed - indexed for MEDLINE]





**Expression of deoxycytidine kinase in leukaemic cells compared with solid tumour cell lines, liver metastases and normal liver.**

**van der Wilt CL, Kroep JR, Loves WJ, Rots MG, Van Groenigen CJ, Kaspers GJ, Peters GJ.**

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Deoxycytidine kinase (dCK) is required for the phosphorylation of several deoxyribonucleoside analogues that are widely employed as chemotherapeutic agents. Examples include cytosine arabinoside (Ara-C) and 2-chlorodeoxyadenosine (CdA) in the treatment of acute myeloid leukaemia (AML) and gemcitabine to treat solid tumours. In this study, expression of dCK mRNA was measured by a competitive template reverse transcriptase polymerase chain reaction (CT RT-PCR) in seven cell lines of different histological origin, 16 childhood and adult AML samples, 10 human liver samples and 11 human liver metastases of colorectal cancer origin. The enzyme activity and protein expression levels of dCK in the cell lines were closely related to the mRNA expression levels ( $r=0.75$ ,  $P=0.026$  and  $r=0.86$ ,  $P=0.007$ ). In AML samples, dCK mRNA expression ranged from 1.16 to 35.25 ( $\times 10^{-3}$ ) dCK/beta-actin. In the cell line panel, the range was 2.97-56.9 ( $\times 10^{-3}$ ) dCK/beta-actin of dCK mRNA expression. The enzyme activity in liver metastases was correlated to dCK mRNA expression ( $r=0.497$ ,  $P=0.05$ ). In the liver samples, these were not correlated. dCK mRNA expression showed only a 36-fold range in liver while a 150-fold range was observed in the liver metastases. In addition, dCK activity and mean mRNA levels were 2.5-fold higher in the metastases than in the liver samples. Since dCK is associated with the sensitivity to deoxynucleoside analogues and because of the good correlation between the different dCK measurements in malignant cells and tumours, the CT-RT PCR assay will be useful in the selection of patients that can be treated with deoxycytidine analogues.

PMID: 12628850 [PubMed - indexed for MEDLINE]

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**Expression of somatostatin receptor types 1-5 in 81 cases of gastrointestinal and pancreatic endocrine tumors. A correlative immunohistochemical and reverse-transcriptase polymerase chain reaction analysis.**

**Papotti M, Bongiovanni M, Volante M, Allia E, Landolfi S, Helboe L, Schindler M, Cole SL, Bussolati G.**

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Somatostatin receptors (SSTRs) have been extensively mapped in human tumors by means of autoradiography, reverse-transcriptase polymerase chain reaction (RT-PCR), in situ hybridization (ISH) and immunohistochemistry (IHC). We analyzed the SSTR type 1-5 expression by means of RT-PCR and/or IHC in a series of 81 functioning and non-functioning gastroenteropancreatic (GEP) endocrine tumors and related normal tissues. Moreover, we compared the results with clinical, pathological and hormonal features. Forty-six cases (13 intestinal and 33 pancreatic) were studied for SSTR 1-5 expression using RT-PCR, IHC with antibodies to SSTR types 2, 3, 5 and ISH for SSTR2 mRNA. The vast majority of tumors expressed SSTR types 1, 2, 3 and 5, while SSTR4 was detected in a small minority. Due to the good correlation between RT-PCR and IHC data on SSTR types 2, 3, and 5, thirty-five additional GEP endocrine tumors were studied with IHC alone. Pancreatic insulinomas had an heterogeneous SSTR expression, while 100% of somatostatinomas expressed SSTR5 and 100% gastrinomas and glucagonomas expressed SSTR2. Pre-operative biopsy material showed an overlapping immunoreactivity with that of surgical specimens, suggesting that the SSTR status can be detected in the diagnostic work-up. It is concluded that SSTRs 1-5 are heterogeneously expressed in GEP endocrine tumors and that IHC is a reliable tool to detect SSTR types 2, 3 and 5 in surgical and biopsy specimens.

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**Overexpression of a DEAD box protein (DDX1) in neuroblastoma and retinoblastoma cell lines.**

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The DEAD box gene, DDX1, is a putative RNA helicase that is co-amplified with MYCN in a subset of retinoblastoma (RB) and neuroblastoma (NB) tumors and cell lines. Although gene amplification usually involves hundreds to thousands of kilobase pairs of DNA, a number of studies suggest that co-amplified genes are only overexpressed if they provide a selective advantage to the cells in which they are amplified. Here, we further characterize DDX1 by identifying its putative transcription and translation initiation sites. We analyze DDX1 protein levels in MYCN/DDX1-amplified NB and RB cell lines using polyclonal antibodies specific to DDX1 and show that there is a good correlation with DDX1 gene copy number, DDX1 transcript levels, and DDX1 protein levels in all cell lines studied. DDX1 protein is found in both the nucleus and cytoplasm of DDX1-amplified lines but is localized primarily to the nucleus of nonamplified cells. Our results indicate that DDX1 may be involved in either the formation or progression of a subset of NB and RB tumors and suggest that DDX1 normally plays a role in the metabolism of RNAs located in the nucleus of the cell.

PMID: 9694872 [PubMed - indexed for MEDLINE]

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266: Mol Cell Biol. 1999 Nov;19(11):7357-68.

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### **A sampling of the yeast proteome.**

**Futcher B, Latter GI, Monardo P, McLaughlin CS, Garrels JJ.**

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In this study, we examined yeast proteins by two-dimensional (2D) gel electrophoresis and gathered quantitative information from about 1,400 spots. We found that there is an enormous range of protein abundance and, for identified spots, a good correlation between protein abundance, mRNA abundance, and codon bias. For each molecule of well-translated mRNA, there were about 4,000 molecules of protein. The relative abundance of proteins was measured in glucose and ethanol media. Protein turnover was examined and found to be insignificant for abundant proteins. Some phosphoproteins were identified. The behavior of proteins in differential centrifugation experiments was examined. Such experiments with 2D gels can give a global view of the yeast proteome.

PMID: 10523624 [PubMed - indexed for MEDLINE]

**Expression of human telomerase reverse transcriptase gene and protein, and of estrogen and progesterone receptors, in breast tumors: Preliminary data from neo-adjuvant chemotherapy.**

**Kammori M, Izumiyama N, Hashimoto M, Nakamura K, Okano T, Kurabayashi R, Naoki H, Honma N, Ogawa T, Kaminishi M, Takubo K.**

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Human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, is very closely associated with telomerase activity. Telomerase has been implicated in cellular immortalization and carcinogenesis. In situ detection of hTERT will aid in determining the localization of telomerase-positive cells. The aim of this study was to detect expression of hTERT mRNA, hTERT protein, estrogen receptor (ER) and progesterone receptor (PR) in paraffin-embedded breast tissue samples and to investigate the relationship between hTERT expression and various clinicopathological parameters in breast tumorigenesis. We used in situ hybridization (ISH) to examine hTERT gene expression, and immunohistochemistry (IHC) to examine expression of hTERT protein, ER and PR, in breast tissues including 64 adenocarcinomas, 2 phyllode tumors and their adjacent normal breast tissues. hTERT gene expression was detected by ISH in 56 (88%) carcinomas, but in neither of the 2 phyllode tumors. hTERT protein expression was detected by IHC in 52 (81%) carcinomas, but in neither of the 2 phyllode tumors. Moreover, ER and PR were expressed in 42 (66%) and 42 (66%) carcinomas, respectively, and in neither of the 2 phyllode tumors. In 4 cases of breast carcinoma that strongly expressed hTERT gene and protein before treatment, neo-adjuvant chemotherapy led to disappearance of gene and protein expression in all cases. There was a strong correlation between detection of hTERT gene expression by ISH and of hTERT protein by ICH in tissue specimens from breast tumors. These results suggest that detection of hTERT protein by ICH can be used to distinguish breast cancers as a potential diagnostic and therapeutic marker.

PMID: 16211220 [PubMed - in process]

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141: Zhonghua Jie He He Hu Xi Za Zhi. 2002 Jun;25(6):337-40.

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**[The pathogenic role of macrophage migration inhibitory factor in acute respiratory distress syndrome]**

[Article in Chinese]

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**OBJECTIVE** To investigate the expression and pathogenic role of macrophage migration inhibitory factor( MIF) in human acute respiratory distress syndrome(ARDS)

**METHODS** The serum level of MIF in ARDS patients and normal persons were measured by ELISA method. Peripheral blood mononuclear cell (PBMC) MIF expression was determined by flow- cytometry. The expression of MIF mRNA and protein in the lung tissues were detected by using double immuno histochemistry labeling and in situ hybridization. **RESULTS** The serum level of MIF increased significantly in ARDS patients as compared with normal persons ( $P < 0.01$ ). The percentage of PBMC MIF expression was higher in ARDS patients than in normal controls ( $P < 0.01$ ). In situ hybridization and immunohistochemistry showed undetectable or weak MIF mRNA and protein expression in normal lungs. In contrast, there was marked upregulation of MIF mRNA and protein expression in the ARDS lungs. In ARDS macro phages infiltrated the alveolar space and interstitium, most of which also expressed MIF. Infiltrating macrophages were almost restricted to the areas of severe tissue damage. The MIF expression level showed a strong correlation with the number of infiltrating macrophages. **CONCLUSIONS** The serum level of MIF and PBMC MIF expression increased in ARDS patients with enhanced pulmonary MIF expression and macrophage infiltration, which suggests that MIF plays a pivotal role in the pathogenesis of ARDS.

PMID: 12126556 [PubMed - indexed for MEDLINE]



**The decompensated detrusor III: impact of bladder outlet obstruction on sarcoplasmic endoplasmic reticulum protein and gene expression.**

**Stein R, Gong C, Hutcheson JC, Canning DA, Zderic SA.**

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**PURPOSE:** Regulation of calcium ion homeostasis has a significant role in smooth muscle contractility. The sarcoplasmic endoplasmic reticulum, calcium, magnesium, adenosine triphosphatase (SERCA) is a regulatory ion pump that may have a role in the functional outcome after outlet obstruction. We investigate what correlation if any existed between SERCA protein and gene expression, and the contractile properties in the same bladder. **MATERIALS AND METHODS:** Standardized partial bladder outlet obstructions were created in adult New Zealand white rabbits, which were divided into control, sham operated and obstructed groups. Muscle strip studies subcategorized the obstructed group into compensated (force greater than 50% of control) and decompensated (force less than 50% of control). Microsomal membrane and total RNA fractions were prepared from the same bladder tissue. Membrane proteins were used for Western blot analysis using a SERCA specific monoclonal antibody, and total RNA was assessed with Northern blot analysis. **RESULTS:** The relative intensities of signals for the Western and Northern blots demonstrated a strong correlation between protein and gene expression. Furthermore there was a strong association between the loss of SERCA messenger RNA and protein expression and loss of bladder function. **CONCLUSIONS:** Bladder contractility after outlet obstruction is influenced in part by smooth muscle cell ability to maintain calcium homeostasis via SERCA. The loss of SERCA protein expression is mediated by down-regulation in gene expression in the same bladder. These data suggest that smooth muscle ion pump gene expression is in part mechanically (pressure work) regulated.

PMID: 10958733 [PubMed - indexed for MEDLINE]

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**Follicle-stimulating hormone receptor and its messenger ribonucleic acid are present in the bovine cervix and can regulate cervical prostanoid synthesis.**

**Mizrachi D, Shemesh M.**

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The hypothesis that FSH regulates the bovine cervical prostaglandin E(2) (PGE(2)) synthesis that is known to be associated with cervical relaxation and opening at the time of estrus was investigated. Cervical tissue from pre-estrous/estrous, luteal, and postovulatory cows were examined for 1) the presence of bovine (b) FSH receptor (R) and its corresponding mRNA and 2) the effect of FSH on the PGE(2) regulatory pathway in vitro. The presence of bFSHR mRNA in the cervix (maximal during pre-estrus/estrus) was demonstrated by the expression of a reverse transcription (RT) polymerase chain reaction (PCR) product (384 base pairs) specific for bFSHR mRNA and sequencing. Northern blotting revealed three transcripts (2.5, 3.3, and 3.8 kilobases [kb]) in cervix from pre-estrous/estrous cows. The level of FSHR (75 kDa) was significantly higher ( $p < 0.01$ ) in Western blots of pre-estrous/estrous cervix than in other cervical tissues. There was a good correlation between the 75-kDa protein expression and its corresponding transcript of 2.55 kb throughout the estrous cycle as described by Northern blot analysis as well as RT-PCR. Incubation of FSH (10 ng/ml) with pre-estrous/estrous cervix resulted in a 3-fold increase in the expression of FSHR and a 2-fold increase in both G protein ( $\alpha(s)$ ) and cyclooxygenase II. FSH (5-20 ng/ml) significantly increased ( $p < 0.01$ ) cAMP, inositol phosphate ( $p < 0.01$ ), and PGE(2) ( $p < 0.01$ ) production by pre-estrous/estrous cervix but not by cervix at the other stages. We conclude that bovine cervix at the time of the peripheral plasma FSH peak (pre-estrus/estrus) contains high levels of FSHR and responds to FSH by increasing the PGE(2) production responsible for cervical relaxation at estrus.

PMID: 10456856 [PubMed - indexed for MEDLINE]





**Alterations in neuropeptide Y levels and Y1 binding sites in the Flinders Sensitive Line rats, a genetic animal model of depression.**

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Previously, we observed specific alterations of neuropeptide Y (NPY) and Y1 receptor mRNA expression in discrete regions of the Flinders Sensitive Line rats (FSL), an animal model of depression. In order to clarify the correlation between mRNA expression and protein content, radioimmunoassay and receptor autoradiography were currently performed. In the FSL rats, NPY-like immunoreactivity (NPY-LI) was decreased in the hippocampal CA region, while Y1 binding sites were increased; NPY-LI was increased in the arcuate nucleus. Fluoxetine treatment elevated NPY-LI in the arcuate and anterior cingulate cortex and increased Y1 binding sites in the medial amygdala and occipital cortex in both strains. No differences were found regarding the Y2 binding sites. The results demonstrate a good correlation between NPY peptide and mRNA expression, and sustain the possible involvement of NPY and Y1 receptors in depression.

PMID: 10327163 [PubMed - indexed for MEDLINE]



**Id-1 and Id-2 are overexpressed in pancreatic cancer and in dysplastic lesions in chronic pancreatitis.**

**Maruyama H, Kleeff J, Wildi S, Friess H, Buchler MW, Israel MA, Korc M.**

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Id proteins antagonize basic helix-loop-helix proteins, inhibit differentiation, and enhance cell proliferation. In this study we compared the expression of Id-1, Id-2, and Id-3 in the normal pancreas, in pancreatic cancer, and in chronic pancreatitis (CP). Northern blot analysis demonstrated that all three Id mRNA species were expressed at high levels in pancreatic cancer samples by comparison with normal or CP samples. Pancreatic cancer cell lines frequently coexpressed all three Ids, exhibiting a good correlation between Id mRNA and protein levels, as determined by immunoblotting with highly specific anti-Id antibodies. Immunohistochemistry using these antibodies demonstrated the presence of faint Id-1 and Id-2 immunostaining in pancreatic ductal cells in the normal pancreas, whereas Id-3 immunoreactivity ranged from weak to strong. In the cancer tissues, many of the cancer cells exhibited abundant Id-1, Id-2, and Id-3 immunoreactivity. Scoring on the basis of percentage of positive cells and intensity of immunostaining indicated that Id-1 and Id-2 were increased significantly in the cancer cells by comparison with the respective controls. Mild to moderate Id immunoreactivity was also seen in the ductal cells in the CP-like areas adjacent to these cells and in the ductal cells of small and interlobular ducts in CP. In contrast, in dysplastic and atypical papillary ducts in CP, Id-1 and Id-2 immunoreactivity was as significantly elevated as in the cancer cells. These findings suggest that increased Id expression may be associated with enhanced proliferative potential of pancreatic cancer cells and of proliferating or dysplastic ductal cells in CP.

PMID: 10487839 [PubMed - indexed for MEDLINE]



**Increased expression of proteasome subunits in skeletal muscle of cancer patients with weight loss.**

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Atrophy of skeletal muscle is common in patients with cancer and results in increased morbidity and mortality. In order to design effective therapy the mechanism by which this occurs needs to be elucidated. Most studies suggest that the ubiquitin-proteasome proteolytic pathway is most important in intracellular proteolysis, although there have been no reports on the activity of this pathway in patients with different extents of weight loss. In this report the expression of the ubiquitin-proteasome pathway in rectus abdominis muscle has been determined in cancer patients with weight loss of 0-34% using a competitive reverse transcriptase polymerase chain reaction to measure expression of mRNA for proteasome subunits C2 and C5, while protein expression has been determined by western blotting. Overall, both C2 and C5 gene expression was increased by about three-fold in skeletal muscle of cachectic cancer patients (average weight loss 14.5 $\pm$ 2.5%), compared with that in patients without weight loss, with or without cancer. The level of gene expression was dependent on the amount of weight loss, increasing maximally for both proteasome subunits in patients with weight loss of 12-19%. Further increases in weight loss reduced expression of mRNA for both proteasome subunits, although it was still elevated in comparison with patients with no weight loss. There was no evidence for an increase in expression at weight losses less than 10%. There was a good correlation between expression of proteasome 20S  $\alpha$  subunits, detected by western blotting, and C2 and C5 mRNA, showing that increased gene expression resulted in increased protein synthesis. Expression of the ubiquitin conjugating enzyme, E2(14k), with weight loss followed a similar pattern to that of proteasome subunits. These results suggest variations in the expression of key components of the ubiquitin-proteasome pathway with weight loss of cancer patients, and suggest that another mechanism of protein degradation must be operative for patients with weight loss less than 10%.

PMID: 16125116 [PubMed - in process]

**Real-time quantitative RT-PCR of cyclin D1 mRNA in mantle cell lymphoma: comparison with FISH and immunohistochemistry.**

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Presence of the balanced translocation t(11;14)(q13;q32) and the consequent overexpression of cyclin D1 found in mantle cell lymphoma (MCL) has been shown to be of important diagnostic value. Although many molecular and immunohistochemical approaches have been applied to analyze cyclin D1 status, correlative studies to compare different methods for the diagnosis of MCL are lacking. In this study, we examined 39 archived paraffin specimens from patients diagnosed with a variety of lymphoproliferative diseases including nine cases meeting morphologic and immunophenotypic criteria for MCL by: (1) real-time quantitative RT-PCR to evaluate cyclin D1 mRNA expression; (2) dual fluorescence in situ hybridization (FISH) to evaluate the t(11;14) translocation in interphase nuclei; and (3) tissue array immunohistochemistry to evaluate the cyclin D1 protein level. Among the nine cases of possible MCL, seven cases showed overexpression of cyclin D1 mRNA (cyclin D1 positive MCL) and two cases showed no cyclin D1 mRNA increase (cyclin D1 negative "MCL-like"). In six of seven cyclin D1 positive cases, the t(11;14) translocation was demonstrated by FISH analysis; in one case FISH was unsuccessful. Six of the seven cyclin D1 mRNA overexpressing cases showed increased cyclin D1 protein on tissue array immunohistochemistry; one was technically suboptimal. Among the two cyclin D1 negative MCL-like cases, FISH confirmed the absence of the t(11;14) translocation in both cases. All other lymphoproliferative diseases studied were found to have low or no cyclin D1 mRNA expression and were easily distinguishable from the cyclin D1 overexpressing MCLs by all three techniques. In addition, to confirming the need to assess cyclin D1 status, as well as, morphology and immunophenotyping to establish the diagnosis of MCL, this study demonstrates good correlation and comparability between measure of cyclin D1 mRNA, the 11;14 translocation and cyclin D1 protein.

Publication Types:

- Evaluation Studies

PMID: 12952233 [PubMed - indexed for MEDLINE]



**Vascular endothelial growth factor expression correlates with matrix metalloproteinases MT1-MMP, MMP-2 and MMP-9 in human glioblastomas.**

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Vascular endothelial growth factor (VEGF) is the major endothelial mitogen in central nervous system neoplasms and it is expressed in 64-95% of glioblastomas (GBMs). Tumour cells are the main source of VEGF in GBMs whereas VEGF receptors (VEGFR-1, its soluble form sVEGFR-1, VEGFR-2 and neuropilin-1) are expressed predominantly by endothelial cells. Infiltrating tumour cells and newly-formed capillaries progress through the extracellular matrix by local proteolysis involving matrix metalloproteinases (MMPs). Recent studies have shown that VEGF expression and bioavailability can be modulated by MMPs. We reported previously that the expression of MT1-MMP in human breast cancer cells was associated with an enhanced VEGF expression. We used quantitative RT-PCR, Western blot, gelatin zymography and immunohistochemistry to study the expression of VEGF, VEGFR-1, VEGFR-2, sVEGFR-1, neuropilin-1, MT1-MMP, MMP-2, MMP-9 and TIMP-2 in 20 human GBMs and 5 normal brains. The expression of these MMPs was markedly increased in most GBMs with excellent correlation between mRNA and protein levels; activated forms of MMP-2 and MMP-9 were present in 8/18 and 7/18 of GBMs. A majority of GBMs (17/20) also expressed high levels of VEGF, as previously reported, with strong correlation between VEGF and MT1-MMP gene expression levels, and double immunostaining showed that VEGF and MT1-MMP peptides co-localize in tumour and endothelial cells. Our results suggest that the interplay between metalloproteinases and VEGF previously described in experimental tumours may also be operative in human GBMs. Because of its dual ability to activate MMP-2 and to up-regulate VEGF, MT1-MMP might be of central importance in the growth of GBMs and represent an interesting target for anti-cancer treatments. Copyright 2003 Wiley-Liss, Inc.

PMID: 12918061 [PubMed - indexed for MEDLINE]



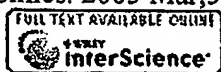
**Expression of cadherins and catenins in paired tumor and non-neoplastic primary prostate cultures and corresponding prostatectomy specimens.**

**Wang J, Krill D, Torbenson M, Wang Q, Bisceglia M, Stoner J, Thomas A, DeFlavia P, Dhir R, Becich MJ.**

Department of Pathology, University of Pittsburgh Medical Center, PA, USA.

Cadherins are a family of transmembrane proteins that play a crucial role in cell differentiation, cell migration, and intercellular adhesion. Cadherins are associated with catenins through their highly conserved cytoplasmic domain. Down-regulation of E-cadherin protein has been shown in various human cancers. This study examined the expression of cadherins and associated catenins at the mRNA level. Paired tumor and nonneoplastic primary prostate cultures were obtained from surgical specimens. Quantitative multiplex fluorescence reverse transcriptase-polymerase chain reaction (QMF RT-PCR) and quantitative analysis were performed and correlated with immunostain results. Six of seven cases of neoplastic cultures showed moderately-to-markedly decreased levels of E-cadherin and P-cadherin mRNA. Similar losses of alpha-catenin and beta-catenin mRNA were also observed. The results of QMF RT-PCR showed good correlation with the results of immunohistochemical studies based on corresponding formalin-fixed sections. In conclusion, this paper presents a coordinated down-regulation in the expression of E-cadherin and associated catenins at the mRNA and protein level in most of the cases studied. This down-regulation may play an important role in the pathogenesis of prostate cancer.

PMID: 11127708 [PubMed - indexed for MEDLINE]



## **A transcriptomic and proteomic analysis of the effect of CpG-ODN on human THP-1 monocytic leukemia cells.**

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The CpG motif of bacterial DNA (CpG-DNA) is a potent immunostimulating agent whose mechanism of action is not yet clear. Here, we used both DNA microarray and proteomic approaches to investigate the effects of oligodeoxynucleotides containing the CpG motif (CpG-ODN) on gene transcription and protein expression profiles of CpG-ODN responsive THP-1 cells. Microarray analysis revealed that 2 h stimulation with CpG-ODN up-regulated 50 genes and down-regulated five genes. These genes were identified as being associated with inflammation, antimicrobial defense, transcriptional regulation, signal transduction, tumor progression, cell differentiation, proteolysis and metabolism. Longer stimulation (8 h) with CpG-ODN enhanced transcriptional expression of 58 genes. Among these 58 genes, none except one, namely WNT1 inducible signaling pathway protein 2, was the same as those induced after 2 h stimulation. Proteomic analysis by two-dimensional gel electrophoresis, followed by mass spectrometry identified several proteins up-regulated by CpG-ODN. These proteins included heat shock proteins, modulators of inflammation, metabolic proteins and energy pathway proteins. Comparison of microarray and proteomic expression profiles showed poor correlation. Use of more reliable and sensitive analyses, such as reverse transcriptase polymerase chain reaction, Western blotting and functional assays, on several genes and proteins, nonetheless, confirmed that there is indeed good correlation between mRNA and protein expression after CpG-ODN treatment. This study also revealed that several anti-apoptotic and neuroprotective related proteins, not previously reported, are activated by CpG-DNA. These findings have extended our knowledge on the activation of cells by CpG-DNA and may contribute to further understanding of mechanisms that link innate immunity with acquired immune response(s).

PMID: 15693060 [PubMed - indexed for MEDLINE]

## SECOND DECLARATION OF PAUL POLAKIS, Ph.D.

I, Paul Polakis, Ph.D., declare and say as follows:

1. I am currently employed by Genentech, Inc. where my job title is Staff Scientist.
2. Since joining Genentech in 1999, one of my primary responsibilities has been leading Genentech's Tumor Antigen Project, which is a large research project with a primary focus on identifying tumor cell markers that find use as targets for both the diagnosis and treatment of cancer in humans.
3. As I stated in my previous Declaration dated May 7, 2004 (attached as Exhibit A), my laboratory has been employing a variety of techniques, including microarray analysis, to identify genes which are differentially expressed in human tumor tissue relative to normal human tissue. The primary purpose of this research is to identify proteins that are abundantly expressed on certain human tumor tissue(s) and that are either (i) not expressed, or (ii) expressed at detectably lower levels, on normal tissue(s).
4. In the course of our research using microarray analysis, we have identified approximately 200 gene transcripts that are present in human tumor tissue at significantly higher levels than in normal human tissue. To date, we have successfully generated antibodies that bind to 31 of the tumor antigen proteins expressed from these differentially expressed gene transcripts and have used these antibodies to quantitatively determine the level of production of these tumor antigen proteins in both human tumor tissue and normal tissue. We have then quantitatively compared the levels of mRNA and protein in both the tumor and normal tissues analyzed. The results of these analyses are attached herewith as Exhibit B. In Exhibit B, "+" means that the mRNA or protein was detectably overexpressed in the tumor tissue relative to normal tissue and "-" means that no detectable overexpression was observed in the tumor tissue relative to normal tissue.
5. As shown in Exhibit B, of the 31 genes identified as being detectably overexpressed in human tumor tissue as compared to normal human tissue at the mRNA level, 28 of them (i.e., greater than 90%) are also detectably overexpressed in human tumor tissue as compared to normal human tissue at the protein level. As such, in the cases where we have been able to quantitatively measure both (i) mRNA and (ii) protein levels in both (i) tumor tissue and (ii) normal tissue, we have observed that in the vast majority of cases, there is a very strong correlation between increases in mRNA expression and increases in the level of protein encoded by that mRNA.



6. Based upon my own experience accumulated in more than 20 years of research, including the data discussed in paragraphs 4-5 above and my knowledge of the relevant scientific literature, it is my considered scientific opinion that for human genes, an increased level of mRNA in a tumor tissue relative to a normal tissue more often than not correlates to a similar increase in abundance of the encoded protein in the tumor tissue relative to the normal tissue. In fact, it remains a generally accepted working assumption in molecular biology that increased mRNA levels are more often than not predictive of elevated levels of the encoded protein. In fact, an entire industry focusing on the research and development of therapeutic antibodies to treat a variety of human diseases, such as cancer, operates on this working assumption.
7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 3-29-00

By: Paul Polakis

Paul Polakis, Ph.D.

DECLARATION OF PAUL POLAKIS, Ph.D.

I, Paul Polakis, Ph.D., declare and say as follows:

1. I was awarded a Ph.D. by the Department of Biochemistry of the Michigan State University in 1984. My scientific Curriculum Vitae is attached to and forms part of this Declaration (Exhibit A).
2. I am currently employed by Genentech, Inc. where my job title is Staff Scientist. Since joining Genentech in 1999, one of my primary responsibilities has been leading Genentech's Tumor Antigen Project, which is a large research project with a primary focus on identifying tumor cell markers that find use as targets for both the diagnosis and treatment of cancer in humans.
3. As part of the Tumor Antigen Project, my laboratory has been analyzing differential expression of various genes in tumor cells relative to normal cells. The purpose of this research is to identify proteins that are abundantly expressed on certain tumor cells and that are either (i) not expressed, or (ii) expressed at lower levels, on corresponding normal cells. We call such differentially expressed proteins "tumor antigen proteins". When such a tumor antigen protein is identified, one can produce an antibody that recognizes and binds to that protein. Such an antibody finds use in the diagnosis of human cancer and may ultimately serve as an effective therapeutic in the treatment of human cancer.
4. In the course of the research conducted by Genentech's Tumor Antigen Project, we have employed a variety of scientific techniques for detecting and studying differential gene expression in human tumor cells relative to normal cells, at genomic DNA, mRNA and protein levels. An important example of one such technique is the well known and widely used technique of microarray analysis which has proven to be extremely useful for the identification of mRNA molecules that are differentially expressed in one tissue or cell type relative to another. In the course of our research using microarray analysis, we have identified approximately 200 gene transcripts that are present in human tumor cells at significantly higher levels than in corresponding normal human cells. To date, we have generated antibodies that bind to about 30 of the tumor antigen proteins expressed from these differentially expressed gene transcripts and have used these antibodies to quantitatively determine the level of production of these tumor antigen proteins in both human cancer cells and corresponding normal cells. We have then compared the levels of mRNA and protein in both the tumor and normal cells analyzed.
5. From the mRNA and protein expression analyses described in paragraph 4 above, we have observed that there is a strong correlation between changes in the level of mRNA present in any particular cell type and the level of protein

expressed from that mRNA in that cell type. In approximately 80% of our observations we have found that increases in the level of a particular mRNA correlates with changes in the level of protein expressed from that mRNA when human tumor cells are compared with their corresponding normal cells.

6. Based upon my own experience accumulated in more than 20 years of research, including the data discussed in paragraphs 4 and 5 above and my knowledge of the relevant scientific literature, it is my considered scientific opinion that for human genes, an increased level of mRNA in a tumor cell relative to a normal cell typically correlates to a similar increase in abundance of the encoded protein in the tumor cell relative to the normal cell. In fact, it remains a central dogma in molecular biology that increased mRNA levels are predictive of corresponding increased levels of the encoded protein. While there have been published reports of genes for which such a correlation does not exist, it is my opinion that such reports are exceptions to the commonly understood general rule that increased mRNA levels are predictive of corresponding increased levels of the encoded protein.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 5/07/04

By: Paul Polakis

Paul Polakis, Ph.D.

## CURRICULUM VITAE

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1997 -1999	Research Director Onyx Pharmaceuticals, Richmond, CA
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1991-1992	Senior Scientist, Chiron Corporation, Emeryville, CA.
1989-1991	Scientist, Cetus Corporation, Emeryville CA.
1987-1989	Postdoctoral Research Associate, Genentech, Inc., South San Francisco, CA.
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**EXHIBIT B**

	tumor mRNA	tumor IHC
UNQ2525	+	+
UNQ2378	+	+
UNQ972	+	-
UNQ97671	+	+
UNQ2964	+	+
UNQ323	+	+
UNQ1655	+	+
UNQ2333	+	+
UNQ9638	+	+
UNQ8209	+	+
UNQ6507	+	+
UNQ8196	+	+
UNQ9109	+	+
UNQ100	+	+
UNQ178	+	+
UNQ1477	+	+
UNQ1839	+	+
UNQ2079	+	+
UNQ8782	+	+
UNQ9646	+	-
UNQ111	+	+
UNQ3079	+	+
UNQ8175	+	+
UNQ9509	+	+
UNQ10978	+	-
UNQ2103	+	+
UNQ1563	+	+
UNQ16188	+	+
UNQ13589	+	+
UNQ1078	+	+
UNQ879	+	+

# Genome-wide Study of Gene Copy Numbers, Transcripts, and Protein Levels in Pairs of Non-invasive and Invasive Human Transitional Cell Carcinomas\*

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Gain and loss of chromosomal material is characteristic of bladder cancer, as well as malignant transformation in general. The consequences of these changes at both the transcription and translation levels is at present unknown partly because of technical limitations. Here we have attempted to address this question in pairs of non-invasive and invasive human bladder tumors using a combination of technology that included comparative genomic hybridization, high density oligonucleotide array-based monitoring of transcript levels (5600 genes), and high resolution two-dimensional gel electrophoresis. The results showed that there is a gene dosage effect that in some cases superimposes on other regulatory mechanisms. This effect depended ( $p < 0.015$ ) on the magnitude of the comparative genomic hybridization change. In general (18 of 23 cases), chromosomal areas with more than 2-fold gain of DNA showed a corresponding increase in mRNA transcripts. Areas with loss of DNA, on the other hand, showed either reduced or unaltered transcript levels. Because most proteins resolved by two-dimensional gels are unknown it was only possible to compare mRNA and protein alterations in relatively few cases of well focused abundant proteins. With few exceptions we found a good correlation ( $p < 0.005$ ) between transcript alterations and protein levels. The implications, as well as limitations, of the approach are discussed. *Molecular & Cellular Proteomics* 1:37-45, 2002.

Aneuploidy is a common feature of most human cancers (1), but little is known about the genome-wide effect of this

phenomenon at both the transcription and translation levels. High throughput array studies of the breast cancer cell line BT474 has suggested that there is a correlation between DNA copy numbers and gene expression in highly amplified areas (2), and studies of individual genes in solid tumors have revealed a good correlation between gene dose and mRNA or protein levels in the case of c-erb-B2, cyclin D1, *ems1*, and N-myc (3-5). However, a high cyclin D1 protein expression has been observed without simultaneous amplification (4), and a low level of c-myc copy number increase was observed without concomitant c-myc protein overexpression (6).

In human bladder tumors, karyotyping, fluorescent *in situ* hybridization, and comparative genomic hybridization (CGH)<sup>1</sup> have revealed chromosomal aberrations that seem to be characteristic of certain stages of disease progression. In the case of non-invasive pTa transitional cell carcinomas (TCCs), this includes loss of chromosome 9 or parts of it, as well as loss of Y in males. In minimally invasive pT1 TCCs, the following alterations have been reported: 2q-, 11p-, 1q+, 11q13+, 17q+, and 20q+ (7-12). It has been suggested that these regions harbor tumor suppressor genes and oncogenes; however, the large chromosomal areas involved often contain many genes, making meaningful predictions of the functional consequences of losses and gains very difficult.

In this investigation we have combined genome-wide technology for detecting genomic gains and losses (CGH) with gene expression profiling techniques (microarrays and proteomics) to determine the effect of gene copy number on transcript and protein levels in pairs of non-invasive and invasive human bladder TCCs.

## EXPERIMENTAL PROCEDURES

**Material**—Bladder tumor biopsies were sampled after informed consent was obtained and after removal of tissue for routine pathology examination. By light microscopy tumors 335 and 532 were staged by an experienced pathologist as pTa (superficial papillary).

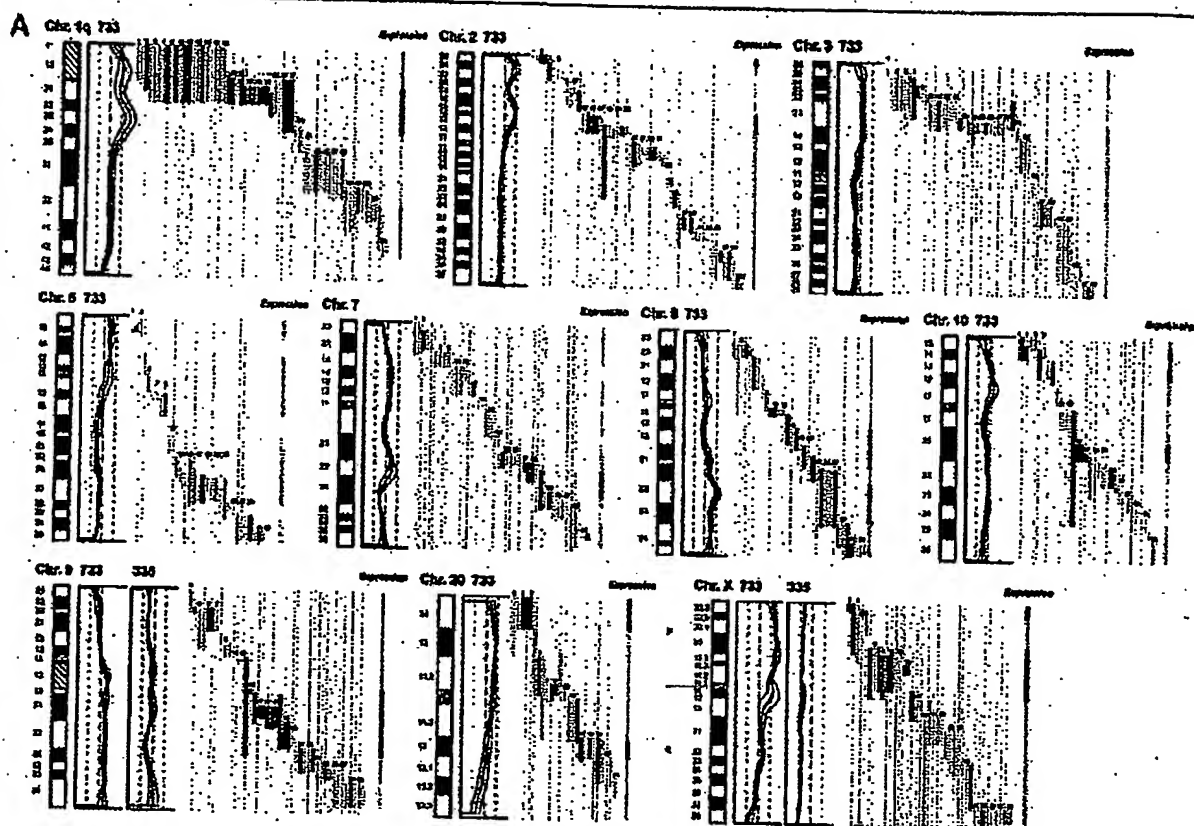
<sup>1</sup> The abbreviations used are: CGH, comparative genomic hybridization; TCC, transitional cell carcinoma; LOH, loss of heterozygosity; PA-FABP, psoriasis-associated fatty acid-binding protein; 2D, two-dimensional.

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Received, September 26, 2001, and in revised form, November 7, 2001

Published, MCP Papers in Press, November 13, 2001, DOI 10.1074/mcp.M100019-MCP200

# Gene Copy Numbers, Transcripts, and Protein Levels



**Fig. 1. DNA copy number and mRNA expression level.** Shown from left to right are chromosome (Chr.), CGH profiles, gene location and expression level of specific genes, and overall expression level along the chromosome. **A**, expression of mRNA in invasive tumor 733 as compared with the non-invasive counterpart tumor 335. **B**, expression of mRNA in invasive tumor 827 compared with the non-invasive counterpart tumor 335. The average fluorescent signal ratio between tumor DNA and normal DNA is shown along the length of the chromosome (left). The bold curve in the ratio profile represents a mean of four chromosomes and is surrounded by thin curves indicating one standard deviation. The central vertical line (broken) indicates a ratio value of 1 (no change), and the vertical lines next to it (dotted) indicate a ratio of 0.5 (left) and 2.0 (right). In chromosomes where the non-invasive tumor 335 used for comparison showed alterations in DNA content, the ratio profile of that chromosome is shown to the right of the invasive tumor profile. The colored bars represent one gene each, identified by the running numbers above the bars (the name of the gene can be seen at [www.MDL.DK/sdata.html](http://www.MDL.DK/sdata.html)). The bars indicate the purported location of the gene, and the colors indicate the expression level of the gene in the invasive tumor compared with the non-invasive counterpart; >2-fold increase (black), >2-fold decrease (blue), no significant change (orange). The bar to the far right, entitled *Expression* shows the resulting change in expression along the chromosome; the colors indicate that at least half of the genes were up-regulated (black), at least half of the genes down-regulated (blue), or more than half of the genes are unchanged (orange). If a gene was absent in one of the samples and present in another, it was regarded as more than a 2-fold change. A 2-fold level was chosen as this corresponded to one standard deviation in a double determination of ~1800 genes. Centromeres and heterochromatic regions were excluded from data analysis.

grade I and II, respectively, tumors 733 and 827 were staged as pT1 (invasive into submucosa), 733 was staged as solid, and 827 was staged as papillary, both grade III.

**mRNA Preparation**—Tissue biopsies, obtained fresh from surgery, were embedded immediately in a sodium-guanidinium thiocyanate solution and stored at  $-80^{\circ}\text{C}$ . Total RNA was isolated using the RNeasy B RNA isolation method (WAK-Chemie Medical GmbH). poly(A)<sup>+</sup> RNA was isolated by an oligo(dT) selection step (Oligotex mRNA kit; Qiagen).

**cRNA Preparation**—1  $\mu\text{g}$  of mRNA was used as starting material. The first and second strand cDNA synthesis was performed using the SuperScript<sup>®</sup> choice system (Invitrogen) according to the manufacturer's instructions but using an oligo(dT) primer containing a T7 RNA polymerase binding site. Labeled cRNA was prepared using the MEGAScript<sup>®</sup> *in vitro* transcription kit (Ambion). Biotin-labeled CTP and

UTP (Enzo) was used, together with unlabeled NTPs in the reaction. Following the *in vitro* transcription reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen).

**Array Hybridization and Scanning**—Array hybridization and scanning was modified from a previous method (13). 10  $\mu\text{g}$  of cRNA was fragmented at  $94^{\circ}\text{C}$  for 35 min in buffer containing 40 mM Tris acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc. Prior to hybridization, the fragmented cRNA in a 6 $\times$  SSPE-T hybridization buffer (1 M NaCl, 10 mM Tris, pH 7.6, 0.005% Triton), was heated to  $95^{\circ}\text{C}$  for 5 min, subsequently cooled to  $40^{\circ}\text{C}$ , and loaded onto the Affymetrix probe array cartridge. The probe array was then incubated for 16 h at  $40^{\circ}\text{C}$  at constant rotation (60 rpm). The probe array was exposed to 10 washes in 6 $\times$  SSPE-T at  $25^{\circ}\text{C}$  followed by 4 washes in 0.5 $\times$  SSPE-T at  $50^{\circ}\text{C}$ . The biotinylated cRNA was stained with a streptavidin-phycoerythrin conjugate, 10  $\mu\text{g}/\text{ml}$  (Molecular Probes) in 6 $\times$  SSPE-T

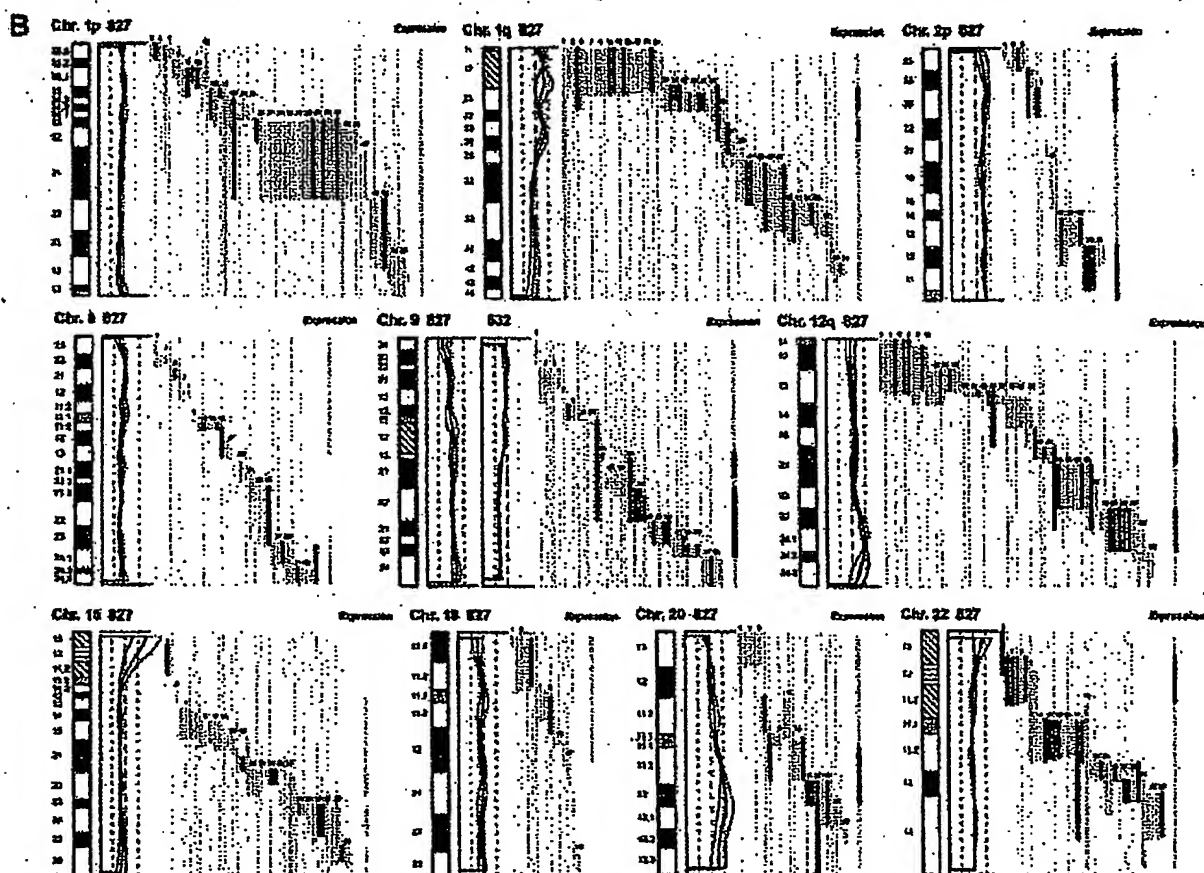


FIG. 1—continued

for 30 min at 25 °C followed by 10 washes in 6× SSPE-T at 25 °C. The probe arrays were scanned at 560 nm using a confocal laser scanning microscope (made for Affymetrix by Hewlett-Packard). The readings from the quantitative scanning were analyzed by Affymetrix gene expression analysis software.

**Microsatellite Analysis**—Microsatellite Analysis was performed as described previously (14). Microsatellites were selected by use of [www.ncbi.nlm.nih.gov/genemap98](http://www.ncbi.nlm.nih.gov/genemap98), and primer sequences were obtained from the genome data base at [www.gdb.org](http://www.gdb.org). DNA was extracted from tumor and blood and amplified by PCR in a volume of 20  $\mu$ l for 35 cycles. The amplicons were denatured and electrophoresed for 3 h in an ABI Prism 377. Data were collected in the Gene Scan program for fragment analysis. Loss of heterozygosity was defined as less than 33% of one allele detected in tumor amplicons compared with blood.

**Proteomic Analysis**—TCCs were minced into small pieces and homogenized in a small glass homogenizer in 0.5 ml of lysis solution. Samples were stored at -20 °C until use. The procedure for 2D gel electrophoresis has been described in detail elsewhere (15, 16). Gels were stained with silver nitrate and/or Coomassie Brilliant Blue. Proteins were identified by a combination of procedures that included microsequencing, mass spectrometry, two-dimensional gel Western immunoblotting, and comparison with the master two-dimensional gel image of human keratinocyte proteins; see [biobase.dk/cgi-bin/cells](http://biobase.dk/cgi-bin/cells).

**CGH**—Hybridization of differentially labeled tumor and normal DNA to normal metaphase chromosomes was performed as described previously (10). Fluorescein-labeled tumor DNA (200 ng), Texas Red-

labeled reference DNA (200 ng), and human Cot-1 DNA (20  $\mu$ g) were denatured at 37 °C for 5 min and applied to denatured normal metaphase slides. Hybridization was at 37 °C for 2 days. After washing, the slides were counterstained with 0.15  $\mu$ g/ml 4,6-diamidino-2-phenylindole in an anti-fade solution. A second hybridization was performed for all tumor samples using fluorescein-labeled reference DNA and Texas Red-labeled tumor DNA (inverse labeling) to confirm the aberrations detected during the initial hybridization. Each CGH experiment also included a normal control hybridization using fluorescein- and Texas Red-labeled normal DNA. Digital image analysis was used to identify chromosomal regions with abnormal fluorescence ratios, indicating regions of DNA gains and losses. The average green:red fluorescence intensity ratio profiles were calculated using four images of each chromosome (eight chromosomes total) with normalization of the green:red fluorescence intensity ratio for the entire metaphase and background correction. Chromosome identification was performed based on 4,6-diamidino-2-phenylindole banding patterns. Only images showing uniform high intensity fluorescence with minimal background staining were analyzed. All centromeres, p arms of acrocentric chromosomes, and heterochromatic regions were excluded from the analysis.

## RESULTS

**Comparative Genomic Hybridization**—The CGH analysis identified a number of chromosomal gains and losses in the

## Gene Copy Numbers, Transcripts, and Protein Levels

TABLE I  
Correlation between alterations detected by CGH and by expression monitoring

Top, CGH used as independent variable (if CGH alteration – what expression ratio was found); bottom, altered expression used as independent variable (if expression alteration – what CGH deviation was found).

CGH alterations	Tumor 733 vs. 335		Concordance	CGH alterations	Tumor 827 vs. 532		Concordance
	Expression change clusters				Expression change clusters		
13 Gain	10 Up-regulation 0 Down-regulation 3 No change		77%	10 Gain	8 Up-regulation 0 Down-regulation 2 No change		80%
10 Loss	1 Up-regulation 6 Down-regulation 4 No change		50%	12 Loss	3 Up-regulation 2 Down-regulation 7 No change		17%
Expression change clusters	Tumor 733 vs. 335		Concordance	Expression change clusters	Tumor 827 vs. 532		Concordance
	CGH alterations				CGH alterations		
16 Up-regulation	11 Gain 2 Loss 3 No change		69%	17 Up-regulation	10 Gain 5 Loss 2 No change		59%
21 Down-regulation	1 Gain 8 Loss 12 No change		38%	9 Down-regulation	0 Gain 3 Loss 6 No change		33%
15 No change	3 Gain 3 Loss 9 No change		60%	21 No change	1 Gain 3 Loss 17 No change		81%

two invasive tumors (stage pT1, TCCs 733 and 827), whereas the two non-invasive papillomas (stage pTa, TCCs 335 and 532) showed only 9p–, 9q22–q33–, and X–, and 7+, 9q–, and Y–, respectively. Both invasive tumors showed changes (1q22–24+, 2q14.1–qter–, 3q12–q13.3–, 6q12–q22–, 9q34+, 11q12–q13+, 17+, and 20q11.2–q12+) that are typical for their disease stage, as well as additional alterations, some of which are shown in Fig. 1. Areas with gains and losses deviated from the normal copy number to some extent, and the average numerical deviation from normal was 0.4-fold in the case of TCC 733 and 0.3-fold for TCC 827. The largest changes, amounting to at least a doubling of chromosomal content, were observed at 1q23 in TCC 733 (Fig. 1A) and 20q12 in TCC 827 (Fig. 1B).

**mRNA Expression in Relation to DNA Copy Number**—The mRNA levels from the two invasive tumors (TCCs 827 and 733) were compared with the two non-invasive counterparts (TCCs 532 and 335). This was done in two separate experiments in which we compared TCCs 733 to 335 and 827 to 532, respectively, using two different scaling settings for the arrays to rule out scaling as a confounding parameter. Approximately 1,800 genes that yielded a signal on the arrays were searched in the Unigene and Genemap data bases for chromosomal location, and those with a known location (1096) were plotted as bars covering their purported locus. In that way it was possible to construct a graphic presentation of DNA copy number and relative mRNA levels along the individual chromosomes (Fig. 1).

For each mRNA a ratio was calculated between the level in the invasive versus the non-invasive counterpart. Bars, which represent chromosomal location of a gene, were color-coded according to the expression ratio, and only differences larger

than 2-fold were regarded as informative (Fig. 1). The density of genes along the chromosomes varied, and areas containing only one gene were excluded from the calculations. The resolution of the CGH method is very low, and some of the outlier data may be because of the fact that the boundaries of the chromosomal aberrations are not known at high resolution.

Two sets of calculations were made from the data. For the first set we used CGH alterations as the independent variable and estimated the frequency of expression alterations in these chromosomal areas. In general, areas with a strong gain of chromosomal material contained a cluster of genes having increased mRNA expression. For example, both chromosomes 1q21–q25, 2p and 9q, showed a relative gain of more than 100% in DNA copy number that was accompanied by increased mRNA expression levels in the two tumor pairs (Fig. 1). In most cases, chromosomal gains detected by CGH were accompanied by an increased level of transcripts in both TCCs 733 (77%) and 827 (80%) (Table I, top). Chromosomal losses, on the other hand, were not accompanied by decreased expression in several cases, and were often registered as having unaltered RNA levels (Table I, top). The inability to detect RNA expression changes in these cases was not because of fewer genes mapping to the lost regions (data not shown).

In the second set of calculations we selected expression alterations above 2-fold as the independent variable and estimated the frequency of CGH alterations in these areas. As above, we found that increased transcript expression correlated with gain of chromosomal material (TCC 733, 69% and TCC 827, 59%), whereas reduced expression was often detected in areas with unaltered CGH ratios (Table I, bottom). Furthermore, as a control we looked at areas with no alter-



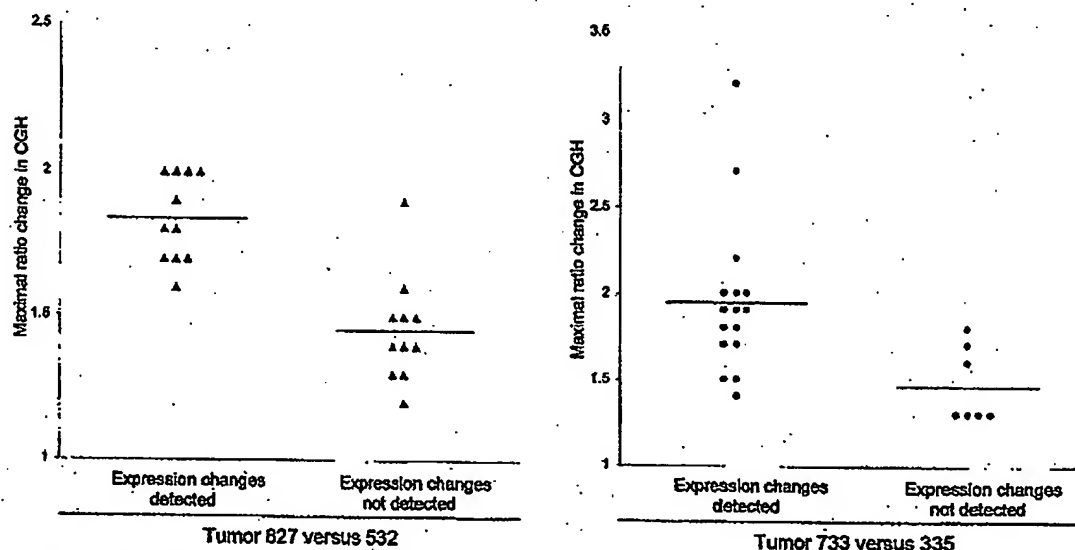


Fig. 2. Correlation between maximum CGH aberration and the ability to detect expression change by oligonucleotide array monitoring. The aberration is shown as a numerical -fold change in ratio between invasive tumors 827 (▲) and 733 (◆) and their non-invasive counterparts 532 and 335. The expression change was taken from the *Expression* line to the right in Fig. 1, which depicts the resulting expression change for a given chromosomal region. At least half of the mRNAs from a given region have to be either up- or down-regulated to be scored as an expression change. All chromosomal arms in which the CGH ratio plus or minus one standard deviation was outside the ratio value of one were included.

ation in expression. No alteration was detected by CGH in most of these areas (TCC 733, 60% and TCC 827, 81%; see Table I, bottom). Because the ability to observe reduced or increased mRNA expression clustering to a certain chromosomal area clearly reflected the extent of copy number changes, we plotted the maximum CGH aberrations in the regions showing CGH changes against the ability to detect a change in mRNA expression as monitored by the oligonucleotide arrays (Fig. 2). For both tumors TCC 733 ( $p < 0.015$ ) and TCC 827 ( $p < 0.00003$ ) a highly significant correlation was observed between the level of CGH ratio change (reflecting the DNA copy number) and alterations detected by the array based technology (Fig. 2). Similar data were obtained when areas with altered expression were used as independent variables. These areas correlated best with CGH when the CGH ratio deviated 1.6- to 2.0-fold (Table I, bottom) but mostly did not at lower CGH deviations. These data probably reflect that loss of an allele may only lead to a 50% reduction in expression level, which is at the cut-off point for detection of expression alterations. Gain of chromosomal material can occur to a much larger extent.

**Microsatellite-based Detection of Minor Areas of Losses**—In TCC 733, several chromosomal areas exhibiting DNA amplification were preceded or followed by areas with a normal CGH but reduced mRNA expression (see Fig. 1, TCC 733 chromosome 1q32, 2p21, and 7q21 and q32, 9q34, and 10q22). To determine whether these results were because of undetected loss of chromosomal material in these regions or

because of other non-structural mechanisms regulating transcription, we examined two microsatellites positioned at chromosome 1q25-32 and two at chromosome 2p22. Loss of heterozygosity (LOH) was found at both 1q25 and at 2p22 indicating that minor deleted areas were not detected with the resolution of CGH (Fig. 3). Additionally, chromosome 2p in TCC 733 showed a CGH pattern of gain/no change/gain of DNA that correlated with transcript increase/decrease/increase. Thus, for the areas showing increased expression there was a correlation with the DNA copy number alterations (Fig. 1A). As indicated above, the mRNA decrease observed in the middle of the chromosomal gain was because of LOH, implying that one of the mechanisms for mRNA down-regulation may be regions that have undergone smaller losses of chromosomal material. However, this cannot be detected with the resolution of the CGH method.

In both TCC 733 and TCC 827, the telomeric end of chromosome 11p showed a normal ratio in the CGH analysis; however, clusters of five and three genes, respectively, lost their expression. Two microsatellites (D11S1760, D11S922) positioned close to MUC2, IGF2, and cathepsin D indicated LOH as the most likely mechanism behind the loss of expression (data not shown).

A reduced expression of mRNA observed in TCC 733 at chromosomes 3q24, 11p11, 12p12.2, 12q21.1, and 16q24 and in TCC 827 at chromosome 11p15.5, 12p11, 15q11.2, and 18q12 was also examined for chromosomal losses using microsatellites positioned as close as possible to the gene loci

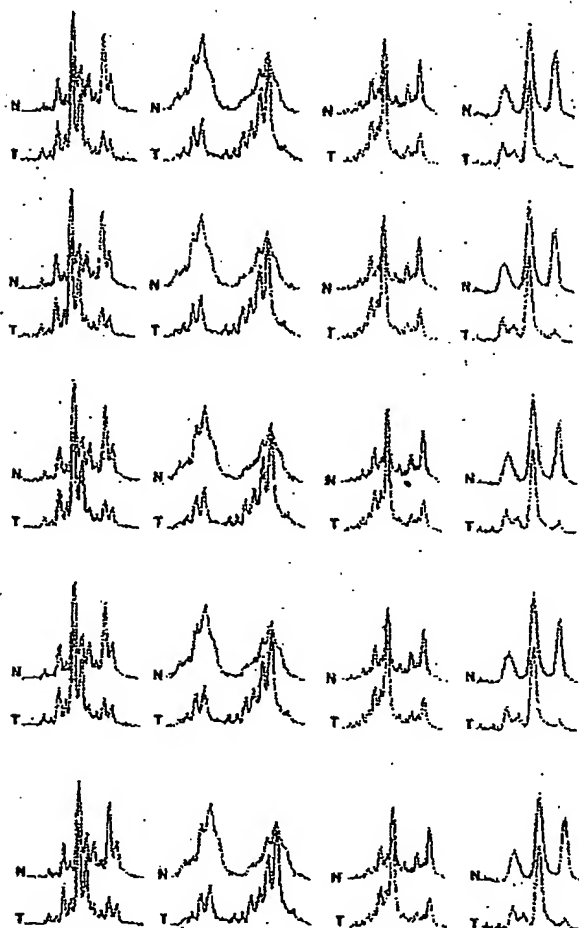


Fig. 3. Microsatellite analysis of loss of heterozygosity. Tumor 733 showing loss of heterozygosity at chromosome 1q25, detected (a) by D1S215 close to HLA class I histocompatibility antigen (gene number 38 in Fig. 1), (b) by D1S2735 close to cathepsin E (gene number 41 in Fig. 1), and (c) at chromosome 2p23 by D2S2251 close to general  $\beta$ -spectrin (gene number 11 on Fig. 1) and of (d) tumor 827 showing loss of heterozygosity at chromosome 18q12 by S18S1118 close to mitochondrial 3-oxoacyl-coenzyme A thiolase (gene number 12 in Fig. 1). The upper curves show the electropherogram obtained from normal DNA from leukocytes (N), and the lower curves show the electropherogram from tumor DNA (T). In all cases one allele is partially lost in the tumor amplicon.

showing reduced mRNA transcripts. Only the microsatellite positioned at 18q12 showed LOH (Fig. 3), suggesting that transcriptional down-regulation of genes in the other regions may be controlled by other mechanisms.

**Relation between Changes in mRNA and Protein Levels—**2D-PAGE analysis, in combination with Coomassie Brilliant Blue and/or silver staining, was carried out on all four tumors using fresh biopsy material. 40 well resolved abundant known proteins migrating in areas away from the edges of the pH

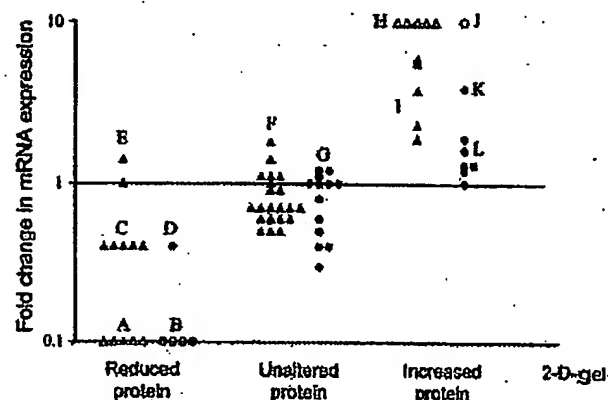
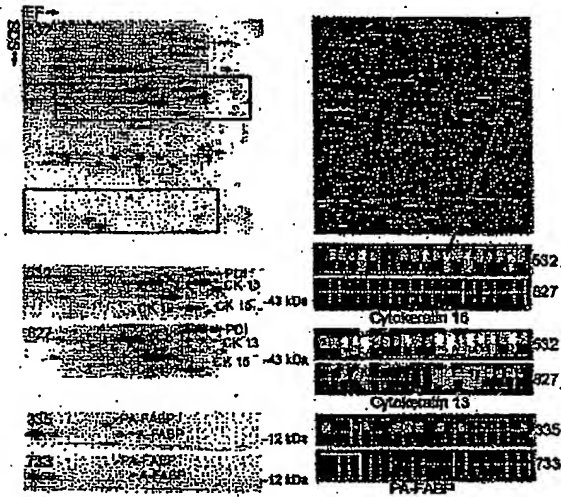


Fig. 4. Correlation between protein levels as judged by 2D-PAGE and transcript ratio. For comparison proteins were divided in three groups, unaltered in level or up- or down-regulated (horizontal axis). The mRNA ratio as determined by oligonucleotide arrays was plotted for each gene (vertical axis). ▲, mRNAs that were scored as present in both tumors used for the ratio calculation; △, mRNAs that were scored as absent in the invasive tumors (along horizontal axis) or as absent in non-invasive reference (top of figure). Two different scalings were used to exclude scaling as a confounder, TCCs 827 and 532 (▲▲) were scaled with background suppression, and TCCs 733 and 335 (●●) were scaled without suppression. Both comparisons showed highly significant ( $p < 0.005$ ) differences in mRNA ratios between the groups. Proteins shown were as follows: Group A (from left), phosphoglucosylase 1, glutathione transferase class  $\mu$  number 4, fatty acid-binding protein homologue, cytokeratin 15, and cytokeratin 13; B (from left), fatty acid-binding protein homologue, 28-kDa heat shock protein, cytokeratin 13, and calyculin; C (from left),  $\alpha$ -enolase, hnRNP B1, 28-kDa heat shock protein, 14-3-3- $\epsilon$ , and pre-mRNA splicing factor; D, mesothelial keratin K7 (type II); E (from top), glutathione S-transferase- $\pi$  and mesothelial keratin K7 (type II); F (from top and left), adenylyl cyclase-associated protein, E-cadherin, keratin 19, calgizarin, phosphoglycerate mutase, annexin IV, cytoskeletal  $\gamma$ -actin, hnRNP A1, integral membrane protein calnexin (P90), hnRNP H, brain-type clathrin light chain- $\alpha$ , hnRNP F, 70-kDa heat shock protein, heterogeneous nuclear ribonucleoprotein A/B, translationally controlled tumor protein, liver glyceraldehyde-3-phosphate dehydrogenase, keratin 8, aldehyde reductase, and Na,K-ATPase  $\beta$ -1 subunit; G, (from top and left), TCP20, calgizarin, 70-kDa heat shock protein, calnexin, hnRNP H, cytokeratin 15, ATP synthase, keratin 19, triosephosphate isomerase, hnRNP F, liver glyceraldehyde-3-phosphate dehydrogenase, glutathione S-transferase- $\pi$ , and keratin 8; H (from left), plasma gelsolin, autoantigen calreticulin, thioredoxin, and NAD $^{+}$ -dependent 15-hydroxyprostaglandin dehydrogenase; I (from top), prollyl 4-hydroxylase  $\beta$ -subunit, cytokeratin 20, cytokeratin 17, prohibitin, and fructose 1,6-bisphosphatase; J annexin II; K, annexin IV; L (from top and left), 90-kDa heat shock protein, prollyl 4-hydroxylase  $\beta$ -subunit,  $\alpha$ -enolase, GRP 78, cyclophilin, and cofilin.

gradient, and having a known chromosomal location, were selected for analysis in the TCC pair 827/532. Proteins were identified by a combination of methods (see "Experimental Procedures"). In general there was a highly significant correlation ( $p < 0.005$ ) between mRNA and protein alterations (Fig. 4). Only one gene showed disagreement between transcript alteration and protein alteration. Except for a group of cyto-



**Fig. 5. Comparison of protein and transcript levels in invasive and non-invasive TCCs.** The upper part of the figure shows a 2D gel (left) and the oligonucleotide array (right) of TCC 532. The red rectangles on the upper gel highlight the areas that are compared below. Identical areas of 2D gels of TCCs 532 and 827 are shown below. Clearly, cytochromes 13 and 15 are strongly down-regulated in TCC 827 (red annotation). The tile on the array containing probes for cytochrome 15 is enlarged below the array (red arrow) from TCC 532 and is compared with TCC 827. The upper row of squares in each tile corresponds to perfect match probes; the lower row corresponds to mismatch probes containing a mutation (used for correction for unspecific binding). Absence of signal is depicted as black, and the higher the signal the lighter the color. A high transcript level was detected in TCC 532 (6161 units) whereas a much lower level was detected in TCC 827 (absence of signals). For cytochrome 13, a high transcript level was also present in TCC 532 (15659 units), and a much lower level was present in TCC 827 (623 units). The 2D gels at the bottom of the figure (left) show levels of PA-FABP and adipocyte-FABP in TCCs 335 and 733 (invasive), respectively. Both proteins are down-regulated in the invasive tumor. To the right we show the array tiles for the PA-FABP transcript. A medium transcript level was detected in the case of TCC 335 (1277 units) whereas very low levels were detected in TCC 733 (166 units). IEF, Isoelectric focusing.

keratins encoded by genes on chromosome 17 (Fig. 5) the analyzed proteins did not belong to a particular family. 26 well focused proteins whose genes had a known chromosomal location were detected in TCCs 733 and 335, and of these 19 correlated ( $p < 0.005$ ) with the mRNA changes detected using the arrays (Fig. 4). For example, PA-FABP was highly expressed in the non-invasive TCC 335 but lost in the invasive counterpart (TCC 733; see Fig. 5). The smaller number of proteins detected in both 733 and 335 was because of the smaller size of the biopsies that were available.

11 chromosomal regions where CGH showed aberrations that corresponded to the changes in transcript levels also showed corresponding changes in the protein level (Table II). These regions included genes that encode proteins that are found to be frequently altered in bladder cancer, namely cytochromes 17 and 20, annexins II and IV, and the fatty acid-binding proteins PA-FABP and FABP1. Four of these proteins were encoded by genes in chromosome 17q, a frequently amplified chromosomal area in invasive bladder cancers.

#### DISCUSSION

Most human cancers have abnormal DNA content, having lost some chromosomal parts and gained others. The present study provides some evidence as to the effect of these gains and losses on gene expression in two pairs of non-invasive and invasive TCCs using high throughput expression arrays and proteomics, in combination with CGH. In general, the results showed that there is a clear individual regulation of the mRNA expression of single genes, which in some cases was superimposed by a DNA copy number effect. In most cases, genes located in chromosomal areas with gains often exhibited increased mRNA expression, whereas areas showing losses showed either no change or a reduced mRNA expression. The latter might be because of the fact that losses most often are restricted to loss of one allele, and the cut-off point for detection of expression alterations was a 2-fold change, thus being at the border of detection. In several cases, how-

**TABLE II**  
Proteins whose expression level correlates with both mRNA and gene dose changes

Protein	Chromosomal location	Tumor TCC	CGH alteration	Transcript alteration <sup>a</sup>	Protein alteration
Annexin II	1q21	733	Gain	Abs to Pres <sup>a</sup>	Increase
Annexin IV	2p13	733	Gain	3.9-Fold up	Increase
Cytokeratin 17	17q12-q21	827	Gain	3.8-Fold up	Increase
Cytokeratin 20	17q21.1	827	Gain	5.6-Fold up	Increase
(PA-)FABP	8q21.2	827	Loss	10-Fold down	Decrease
FABP1	9q22	827	Gain	2.3-Fold up	Increase
Plasma gelsolin	9q31	827	Gain	Abs to Pres	Increase
Heat shock protein 28	15q12-q13	827	Loss	2.5-Fold up	Decrease
Prohibitin	17q21	827/733	Gain	3.7-/2.5-Fold up <sup>b</sup>	Increase
Prolyl-4-hydroxyl	17q25	827/733	Gain	5.7-/1.6-Fold up	Increase
hnRNPB1	7p15	827	Loss	2.5-Fold down	Decrease

<sup>a</sup> Abs, absent; Pres, present.

<sup>b</sup> In cases where the corresponding alterations were found in both TCCs 827 and 733 these are shown as 827/733.

ever, an increase or decrease in DNA copy number was associated with *de novo* occurrence or complete loss of transcript, respectively. Some of these transcripts could not be detected in the non-invasive tumor but were present at relatively high levels in areas with DNA amplifications in the invasive tumors (e.g. in TCC 733 transcript from cellular ligand of annexin II gene (chromosome 1q21) from absent to 2670 arbitrary units; in TCC 827 transcript from small proline-rich protein 1 gene (chromosome 1q12-q21.1) from absent to 1326 arbitrary units). It may be anticipated from these data that significant clustering of genes with an increased expression to a certain chromosomal area indicates an increased likelihood of gain of chromosomal material in this area.

Considering the many possible regulatory mechanisms acting at the level of transcription, it seems striking that the gene dose effects were so clearly detectable in gained areas. One hypothetical explanation may lie in the loss of controlled methylation in tumor cells (17-19). Thus, it may be possible that in chromosomes with increased DNA copy numbers two or more alleles could be demethylated simultaneously leading to a higher transcription level, whereas in chromosomes with losses the remaining allele could be partly methylated, turning off the process (20, 21). A recent report has documented a ploidy regulation of gene expression in yeast, but in this case all the genes were present in the same ratio (22), a situation that is not analogous to that of cancer cells, which show marked chromosomal aberrations, as well as gene dosage effects.

Several CGH studies of bladder cancer have shown that some chromosomal aberrations are common at certain stages of disease progression, often occurring in more than 1 of 3 tumors. In pTa tumors, these include 9p-, 9q-, 1q+, Y- (2, 6), and in pT1 tumors, 2q-, 11p-, 11q-, 1q+, 5p+, 8q+, 17q+, and 20q+ (2-4, 6, 7). The pTa tumors studied here showed similar aberrations such as 9p- and 9q22-q33- and 9q- and Y-, respectively. Likewise, the two minimal invasive pT1 tumors showed aberrations that are commonly seen at that stage, and TCC 827 had a remarkable resemblance to the commonly seen pattern of losses and gains, such as 1q22-24 amplification (seen in both tumors), 11q14-q22 loss, the latter often linked to 17q+ (both tumors), and 1q+ and 9p-, often linked to 20q+ and 11q13+ (both tumors) (7-9). These observations indicate that the pairs of tumors used in this study exhibit chromosomal changes observed in many tumors, and therefore the findings could be of general importance for bladder cancer.

Considering that the mapping resolution of CGH is of about 20 megabases it is only possible to get a crude picture of chromosomal instability using this technique. Occasionally, we observed reduced transcript levels close to or inside regions with increased copy numbers. Analysis of these regions by positioning heterozygous microsatellites as close as possible to the locus showing reduced gene expression revealed loss of heterozygosity in several cases. It seems likely that multiple and different events occur along each chromosomal

arm and that the use of cDNA microarrays for analysis of DNA copy number changes will reach a resolution that can resolve these changes, as has recently been proposed (2). The outlier data were not more frequent at the boundaries of the CGH aberrations. At present we do not know the mechanism behind chromosomal aneuploidy and cannot predict whether chromosomal gains will be transcribed to a larger extent than the two native alleles. A mechanism as genetic imprinting has an impact on the expression level in normal cells and is often reduced in tumors. However, the relation between imprinting and gain of chromosomal material is not known.

We regard it as a strength of this investigation that we were able to compare invasive tumors to benign tumors rather than to normal urothelium, as the tumors studied were biologically very close and probably may represent successive steps in the progression of bladder cancer. Despite the limited amount of fresh tissue available it was possible to apply three different state of the art methods. The observed correlation between DNA copy number and mRNA expression is remarkable when one considers that different pieces of the tumor biopsies were used for the different sets of experiments. This indicates that bladder tumors are relatively homogenous, a notion recently supported by CGH and LOH data that showed a remarkable similarity even between tumors and distant metastasis (10, 23).

In the few cases analyzed, mRNA and protein levels showed a striking correspondence although in some cases we found discrepancies that may be attributed to translational regulation, post-translational processing, protein degradation, or a combination of these. Some transcripts belong to undertranslated mRNA pools, which are associated with few translationally inactive ribosomes; these pools, however, seem to be rare (24). Protein degradation, for example, may be very important in the case of polypeptides with a short half-life (e.g. signaling proteins). A poor correlation between mRNA and protein levels was found in liver cells as determined by arrays and 2D-PAGE (25), and a moderate correlation was recently reported by Ideker et al. (26) in yeast.

Interestingly, our study revealed a much better correlation between gained chromosomal areas and increased mRNA levels than between loss of chromosomal areas and reduced mRNA levels. In general, the level of CGH change determined the ability to detect a change in transcript. One possible explanation could be that by losing one allele the change in mRNA level is not so dramatic as compared with gain of material, which can be rather unlimited and may lead to a severalfold increase in gene copy number resulting in a much higher impact on transcript level. The latter would be much easier to detect on the expression arrays as the cut-off point was placed at a 2-fold level so as not to be biased by noise on the array. Construction of arrays with a better signal to noise ratio may in the future allow detection of lesser than 2-fold alterations in transcript levels, a feature that may facilitate the analysis of the effect of loss of chromosomal areas on transcript levels.

In eleven cases we found a significant correlation between DNA copy number, mRNA expression, and protein level. Four of these proteins were encoded by genes located at a frequently amplified area in chromosome 17q. Whether DNA copy number is one of the mechanisms behind alteration of these eleven proteins is at present unknown and will have to be proved by other methods using a larger number of samples. One factor making such studies complicated is the large extent of protein modification that occurs after translation, requiring immunoidentification and/or mass spectrometry to correctly identify the proteins in the gels.

In conclusion, the results presented in this study exemplify the large body of knowledge that may be possible to gather in the future by combining state of the art techniques that follow the pathway from DNA to protein (26). Here, we used a traditional chromosomal CGH method, but in the future high resolution CGH based on microarrays with many thousand radiation hybrid-mapped genes will increase the resolution and information derived from these types of experiments (2). Combined with expression arrays analyzing transcripts derived from genes with known locations, and 2D gel analysis to obtain information at the post-translational level, a clearer and more developed understanding of the tumor genome will be forthcoming.

**Acknowledgments**—We thank Mle Madsen, Hanne Steen, Inge Lis Thorsen, Hans Lund, Vikolaj Ørntoft, and Lynn Bjerke for technical help and Thomas Gingeras, Christine Harrington, and Morten Østergaard for valuable discussions.

\* This work was supported by grants from The Danish Cancer Society, the University of Aarhus, Aarhus County, Novo Nordic, the Danish Biotechnology Program, the Frønkels Foundation, the John and Birthe Meyer Foundation, and NCI, National Institutes of Health Grant CA47537. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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